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PROVISIONAL APPLICATION TRANSMITTAL SHEET

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Sir:

Transmitted herewith for filing is a provisional patent application under CFR §1.53(c) of Inventors:

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Title: FVII or FVIIa Variants Having Decreased Affinity for TFPI

Enclosed are:

- ☒ 81 pages of the application (including description and claims).
☒ 1 sheet(s) of drawing(s).
☒ Sequence Listing (4 pgs)
☒ 75 claims.
☐ Small entity status is claimed under 37 CFR 1.9(f)
☒ Cover Sheet.
☒ Patent Application Filing Acknowledgement postcard.
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**PROVISIONAL
PATENT APPLICATION**

FVII OR FVIIa VARIANTS HAVING DECREASED AFFINITY FOR TFPI

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FVII OR FVIIa VARIANTS HAVING DECREASED AFFINITY FOR TFPI

FIELD OF THE INVENTION

The present invention relates to novel FVII or FVIIa variants comprising at least one
5 modification in a position selected from the group consisting of 196, 237 and 341. The present invention also relates to use of such polypeptide variants in therapy, in particular for the treatment of a variety of coagulation-related disorders.

BACKGROUND OF THE INVENTION

10 Blood coagulation is a process consisting of a complex interaction of various blood components (or factors) that eventually results in a fibrin clot. Generally, the blood components participating in what has been referred to as the "coagulation cascade" are proenzymes or zymogens, i.e. enzymatically inactive proteins that are converted into an active form by the action of an activator. One of these coagulation factors is factor VII (FVII).

15 FVII is a vitamin K-dependent plasma protein synthesized in the liver and secreted into the blood as a single-chain glycoprotein with a molecular weight of 53 kDa (Broze & Majerus, J. Biol. Chem 1980; 255:1242-1247). The FVII zymogen is converted into an activated form (FVIIa) by proteolytic cleavage at a single site, R152-I153, resulting in two chains linked by a single disulfide bridge. FVIIa in complex with tissue factor (TF), the FVIIa complex, is able to
20 convert both FIX and FX into their activated forms, followed by reactions leading to rapid thrombin production and fibrin formation (Østerud & Rapaport, Proc Natl Acad Sci USA 1977; 74:5260-5264).

FVII undergoes post-translational modifications, including vitamin K-dependent carboxylation resulting in ten γ -carboxyglutamic acid residues in the N-terminal region of the
25 molecule. Thus, residue number 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 shown in SEQ ID NO:1 are γ -carboxyglutamic acids residues in the Gla domain important for FVII activity. Other post-translational modifications include sugar moiety attachment at two naturally occurring N-glycosylation sites at position 145 and 322, respectively, and at two naturally occurring O-glycosylation sites at position 52 and 60, respectively.

The gene coding for human FVII (hFVII) has been mapped to chromosome 13 at q34-
qter 9 (de Grouchy et al., Hum Genet 1984; 66:230-233). It contains nine exons and spans 12.8
Kb (O'Hara et al., Proc Natl Acad Sci USA 1987; 84:5158-5162). The gene organisation and
protein structure of FVII are similar to those of other vitamin K-dependent procoagulant
5 proteins, with exons 1a and 1b encoding for signal sequence; exon 2 the propeptide and Gla
domain; exon 3 a short hydrophobic region; exons 4 and 5 the epidermal growth factor-like
domains; and exon 6 through 8 the serine protease catalytic domain (Yoshitake et al.,
Biochemistry 1985; 24: 3736-3750).

Reports exist on experimental three-dimensional structures of hFVIIa (Pike et al.,
10 PNAS. U.S.A., 1999; 96:8925-30 and Kembell-Cook et al., J.Struct.Biol, 1999; 127:213-223), of
hFVIIa in complex with soluble tissue factor using X-ray crystallographic methods (Banner et
al., Nature, 1996; 380:41 and Zhang et al., J.Mol.Biol, 1999; 285: 2089), and of smaller
fragments of hFVII (Muranyi et al., Biochemistry, 1998; 37:10605 and Kao et al., Biochemistry,
1999; 38:7097).

15 Relatively few protein-engineered variants of FVII have been reported (Dickinson &
Ruf, J Biol Chem, 1997;272:19875-19879, Kembell-Cook et al., J Biol Chem, 1998; 273:8516-
8521, Bharadwaj et al., J Biol Chem, 1996; 271:30685-30691; Ruf et al., Biochemistry, 1999;
38:1957-1966).

Reports exist on expression of FVII in BHK or other mammalian cells (WO 92/15686,
20 WO 91/11514 and WO 88/10295) and co-expression of FVII and kex2 endoprotease in
eukaryotic cells (WO 00/28065).

Commercial preparations of recombinant human FVIIa (rhFVIIa) are sold under the
tradename NovoSeven®. NovoSeven® is indicated for the treatment of bleeding episodes in
hemophilia A or B patients. NovoSeven® is the only rhFVIIa for effective and reliable treatment
25 of bleeding episodes available on the market.

An inactive form of FVII in which arginine 152 and/or isoleucine 153 is/are modified
has been reported in WO 91/11514. These amino acids are located at the activation site. WO
96/12800 describes inactivation of FVIIa by a serine proteinase inhibitor; inactivation by
carbamylation of FVIIa at the α -amino acid group I153 has been described by Petersen et al.,
30 Eur J Biochem, 1999;261:124-129. The inactivated form is capable of competing with hFVII or

hFVIIa for binding to TF and inhibiting clotting activity. The inactivated form of FVIIa is suggested to be used for treatment of patients being in hypercoagulable states, such as patients with sepsis, in risk of myocardial infarction or of thrombotic stroke.

WO 98/32466 suggests that FVII, among many other proteins, may be PEGylated but
5 does not contain any further information in this respect.

WO 01/58935 discloses a new strategy for developing FVII or FVIIa molecules having
inter alia an increased half-life.

A circulating rhFVIIa half-life of 2.3 hours was reported in "Summary Basis for
Approval for NovoSeven®", FDA reference number 96-0597. Relatively high doses and
10 frequent administration are necessary to reach and sustain the desired therapeutic or prophylactic
effect. As a consequence adequate dose regulation is difficult to obtain and the need of frequent
intravenous administrations imposes restrictions on the patient's way of living.

In normal hemostasis, the procoagulant system is in balance with anticoagulant systems
involved in the termination of the hemostatic reaction and the fibrinolytic system, which
15 dissolves clots once they are formed. The anticoagulant systems contain several protease
inhibitors, e.g., the Tissue Factor Pathway Inhibitor (TFPI), antithrombin-III (AT-III), heparin
cofactor-II (HC-II), and the protein C pathway.

TFPI is a reversible, active site-directed inhibitor of FXa, which regulates coagulation
by inhibiting FVIIa-TF in a FXa-dependent manner. The TFPI-FXa complex binds to the FVIIa-
20 TF complex, resulting in the formation of a TF-FVIIa-TFPI-FXa complex.

The *in vivo* relevance of TFPI is supported by experiments showing a hemostatic effect
of a neutralizing anti-TFPI antibody in a hemophila bleeding model (Erhardtsen et al. Blood
Coagul Fibrinolysis 1995; 6:388-394). Furthermore, in biochemical reconstitution experiments,
TFPI was shown to extend the initiation phase and reduce the rate of thrombin generation during
25 the propagation phase (van't Veer and Mann; J. Biol. Chem. 1997; 272: 4367-4377).

It is contemplated that FVIIa variants with reduced sensitivity (reduced affinity) to TFPI
will induce a larger and faster thrombin generation compared to rhFVIIa. This will lead to the
formation of a blood clot with increased clot strength and thus lead to a reduced risk of re-
bleeding. It is also expected that lower doses of a variant of the invention may be administered as
30 the variant is not inhibited by TFPI during circulation.

Accordingly, a first object of the present invention is to provide FVII or FVIIa variants which have a decreased affinity for TFPI as compared to hFVIIa or rhFVIIa. Another object of the present invention is to provide FVII or FVIIa variants which exhibit an increased clotting activity as compared to hFVIIa or rhFVIIa.

5 Another problem in current rhFVIIa treatment is the relative instability of the molecule with respect to proteolytic degradation. Proteolytic degradation is a major obstacle for obtaining a preparation in solution as opposed to a lyophilized product. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient, and, in the case of emergencies, quicker action, which potentially can become life saving. Attempts to prevent proteolytic
10 degradation by site directed mutagenesis at major proteolytic sites have been disclosed in WO 88/10295.

Thus, a further object of the present invention is to provide FVII/FVIIa variants which, in addition to the above-mentioned improved properties, are more stable towards proteolytic degradation, i.e. possess reduced sensitivity to proteolytic degradation.

15 A molecule with a longer circulation half-life would decrease the number of necessary administrations. Given the association of current FVIIa product with frequent injections, and the potential for obtaining more optimal therapeutic FVIIa levels with concomitant enhanced therapeutic effect, there is a clear need for improved FVII- or FVIIa-like molecules. One way to increase the circulation half-life of a protein is to ensure that renal clearance of the protein is
20 reduced. This may be achieved by conjugating the protein to a chemical moiety which is capable of conferring reduced renal clearance to the protein. Furthermore, attachment of a chemical moiety to the protein or substitution of amino acids exposed to proteolysis may effectively block a proteolytic enzyme from contact leading to proteolytic degradation of the protein. Polyethylene glycol (PEG) is one such chemical moiety that has been used in the preparation of therapeutic
25 protein products.

Thus, a further objective of the present invention is to provide FVII/FVIIa variants which, in addition to the above-mentioned improved properties, possess an increased functional *in vivo* half-life and/or an increased serum half-life.

The above-mentioned objectives are met by the improved FVII/FVIIa variants disclosed
30 herein.

BRIEF DISCLOSURE OF THE INVENTION

The present invention provides improved recombinant FVII or FVIIa variants comprising at least one modification in a position selected from the group consisting of 196, 237 and 341. These amino acid modifications result in a decreased binding of FVIIa to TFPI. As indicated above, the resulting molecules have one or more improved properties as compared to commercially available rhFVIIa, such as NovoSeven®.

In interesting embodiments, the FVII or FVIIa variant has been further modified so that the resulting variant has an enhanced phospholipid membrane binding affinity, increased functional *in vivo* half-life, increased plasma half-life and/or an increased Area Under the Curve when administered intravenous (AUC_{iv}). Consequently, medical treatment with such a variant offers a number of advantages over the currently available rhFVIIa compound, such as lower dosage, faster action in uncontrolled bleedings and, optionally, longer duration between injections.

Accordingly, in a first aspect the invention relates to a variant of FVII or FVIIa, wherein said variant comprises at least one modification in a position selected from the group consisting of 196, 237 and 341 as compared to hFVII or hFVIIa (SEQ ID NO:1).

Another aspect of the invention relates to a nucleotide sequence encoding the variant of the invention.

In a further aspect the invention relates to an expression vector comprising the nucleotide sequence of the invention.

In a still further aspect the invention relates to a host cell comprising the nucleotide sequence of the invention or the expression vector of the invention.

In an even further aspect the invention relates to a pharmaceutical composition comprising the variant of the invention, and a pharmaceutically acceptable carrier or excipient.

Still another aspect of the invention relates to a variant of the invention, or a pharmaceutical composition of the invention, for use as a medicament.

Further aspects of the present invention will be apparent from the below description as well as from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the clotting time vs. concentration for [G237GAA]rhFVIIa when assayed in the "Whole Blood Assay". For comparison, the result for rhFVIIa is included. • rhFVIIa;
□ [G237GAA]rhFVIIa.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

In the context of the present application and invention the following definitions apply:

10 The term "conjugate" (or interchangeably "conjugated polypeptide variant") is intended to indicate a heterogeneous (in the sense of composite or chimeric) molecule formed by the covalent attachment of one or more polypeptide(s) to one or more non-polypeptide moieties such as polymer molecules, lipophilic compounds, sugar moieties or organic derivatizing agents. Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e. soluble in
15 physiological fluids such as blood. Examples of conjugated polypeptide variants of the invention include glycosylated and/or PEGylated polypeptides.

The term "covalent attachment" or "covalently attached" means that the polypeptide variant and the non-polypeptide moiety are either directly covalently joined to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties,
20 such as a bridge, spacer, or linkage moiety or moieties.

When used herein, the term "non-polypeptide moiety" means a molecule that is capable of conjugating to an attachment group of the polypeptide variant of the invention. Preferred examples of such molecules include polymer molecules, sugar moieties, lipophilic compounds, or organic derivatizing agents, in particular sugar moieties. When used in the context of a
25 polypeptide variant of the invention it will be understood that the non-polypeptide moiety is linked to the polypeptide part of the polypeptide variant through an attachment group of the polypeptide variant. As explained above, the non-polypeptide moiety may be directly covalently joined to the attachment group or it may be indirectly covalently joined to the attachment group through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or
30 moieties.

The "polymer molecule" is a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is an amino acid residue, except where the polymer is human albumin or another abundant plasma protein. The term "polymer" may be used interchangeably with the term "polymer molecule". The term is also intended to cover carbohydrate molecules attached by *in vitro* glycosylation, i.e. a synthetic glycosylation performed *in vitro* normally involving covalently linking a carbohydrate molecule to an attachment group of the polypeptide variant, optionally using a cross-linking agent. *In vitro* glycosylation is discussed in detail further below.

The term "sugar moiety" is intended to indicate a carbohydrate-containing molecule comprising one or more monosaccharide residues, capable of being attached to the polypeptide variant (to produce a polypeptide variant conjugate in the form of a glycosylated polypeptide variant) by way of *in vivo* glycosylation. The term "*in vivo* glycosylation" is intended to mean any attachment of a sugar moiety occurring *in vivo*, i.e. during posttranslational processing in a glycosylating cell used for expression of the polypeptide variant, e.g. by way of N-linked or O-linked glycosylation. The exact oligosaccharide structure depends, to a large extent, on the glycosylating organism in question.

An "N-glycosylation site" has the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine. Preferably, the amino acid residue in position +3 relative to the asparagine residue is not a proline residue.

An "O-glycosylation site" is the OH-group of a serine or threonine residue.

The term "attachment group" is intended to indicate a functional group of the polypeptide variant, in particular of an amino acid residue thereof or a carbohydrate moiety, capable of attaching a non-polypeptide moiety such as a polymer molecule, a lipophilic molecule, a sugar moiety or an organic derivatizing agent. Useful attachment groups and their matching non-polypeptide moieties are apparent from the table below.

Attachment group	Amino acid	Examples of non-polypeptide moiety	Conjugation method/- Activated PEG	Reference
-NH ₂	N-terminal, Lys	Polymer, e.g. PEG, with amide or imine group	mPEG-SPA Tresylated mPEG	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3,4):249-304 (1992)
-COOH	C-terminal, Asp, Glu	Polymer, e.g. PEG, with ester or amide group Carbohydrate moiety	mPEG-Hz <i>In vitro</i> coupling	Shearwater Inc.
-SH	Cys	Polymer, e.g. PEG, with disulfide, maleimide or vinyl sulfone group Carbohydrate moiety	PEG-vinylsulphone PEG-maleimide <i>In vitro</i> coupling	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3,4):249-304 (1992)
-OH	Ser, Thr, Lys, OH-	Sugar moiety PEG with ester, ether, carbamate, carbonate	<i>In vivo</i> O-linked glycosylation	
-CONH ₂	Asn as part of an N-glycosylation site	Sugar moiety Polymer, e.g. PEG	<i>In vivo</i> N-glycosylation	
Aromatic residue	Phe, Tyr, Trp	Carbohydrate moiety	<i>In vitro</i> coupling	
-CONH ₂	Gln	Carbohydrate moiety	<i>In vitro</i> coupling	Yan and Wold, Biochemistry, 1984, Jul 31; 23(16): 3759-65
Aldehyde Ketone	Oxidized oligo-saccharide	Polymer, e.g. PEG, PEG-hydrazide	PEGylation	Andresz et al., 1978, Makromol. Chem. 179:301, WO 92/16555, WO 00/23114

Guanidino	Arg	Carbohydrate moiety	<i>In vitro</i> coupling	Lundblad and Noyes, Chemical Reagents for Protein Modification, CRC Press Inc., Florida, USA
Imidazole ring	His	Carbohydrate moiety	<i>In vitro</i> coupling	As for guanidine

For *in vivo* N-glycosylation, the term "attachment group" is used in an unconventional way to indicate the amino acid residues constituting a N-glycosylation site (with the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine).

Although the asparagine residue of the N-glycosylation site is the one to which the sugar moiety is attached during glycosylation, such attachment cannot be achieved unless the other amino acid residues of the N-glycosylation site are present. Accordingly, when the non-polypeptide moiety is a sugar moiety and the conjugation is to be achieved by *in vivo* N-glycosylation, the term "amino acid residue comprising an attachment group for the non-polypeptide moiety" as used in connection with alterations of the amino acid sequence of the polypeptide variant is to be understood as meaning that one or more amino acid residues constituting an *in vivo* N-glycosylation site are to be altered in such a manner that either a functional *in vivo* N-glycosylation site is introduced into the amino acid sequence or removed from said sequence.

In the present application, amino acid names and atom names (e.g. CA, CB, CD, CG, SG, NZ, N, O, C, etc) are used as defined by the Protein DataBank (PDB) (www.pdb.org) based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names, etc.), *Eur. J. Biochem.*, 138, 9-37 (1984) together with their corrections in *Eur. J. Biochem.*, 152, 1 (1985)).

The term "amino acid residue" is intended to indicate an amino acid residue contained in the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues.

The terminology used for identifying amino acid positions is illustrated as follows:

G124 indicates that position 124 is occupied by a glycine residue in the amino acid sequence shown in SEQ ID NO:1. G124R indicates that the glycine residue of position 124 has been substituted with an arginine residue. Alternative substitutions are indicated with a "/", e.g.

5. N145S/T means an amino acid sequence in which asparagine in position 145 is substituted with either serine or threonine. Multiple substitutions are indicated with a "+", e.g. K143N+N145S/T means an amino acid sequence which comprises a substitution of the lysine residue in position 143 with an asparagine residue and a substitution of the asparagine residue in position 145 with a serine or a threonine residue. Insertion of an additional amino acid residue, such as insertion of an alanine residue after G124 is indicated by G124GA. Insertion of two additional alanine residues after G124 is indicated by G124GAA, etc. When used herein, the term "inserted in position X" or "inserted at position X" means that the amino acid residue(s) is (are) inserted between amino acid residue X and X+1. A deletion of an amino acid residue is indicated by an asterix. For example, deletion of the glycine residue in position 124 is indicated by G124*.
- 15 Unless otherwise indicated, the numbering of amino acid residues made herein is made relative to the amino acid sequence of hFVII/hFVIIa (SEQ ID NO:1).

The term "differs from" as used in connection with specific mutations is intended to allow for additional differences being present apart from the specified amino acid difference. For instance, in addition to the specified modifications in positions 196, 237 and 341 (aiming at decreasing the affinity for TFPI), the FVII or FVIIa polypeptide variant may comprise other substitutions. Examples of such additional modifications or differences may include truncation of the N- and/or C-terminus by one or more amino acid residues (e.g. by 1-10 amino acid residues), or addition of one or more extra residues at the N- and/or C-terminus, e.g. addition of a methionine residue at the N-terminus or addition of a cysteine residue near or at the C-terminus, as well as "conservative amino acid substitutions", i.e. substitutions performed within groups of amino acids with similar characteristics, e.g. small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids.

25

Examples of such conservative substitutions are shown in the below table.

1.	Alanine (A)	Glycine (G)	Serine (S)	Threonine (T)
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Histidine (H)	Lysine (K)	
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

Still other examples of additional modifications include modifications giving rise to an increased functional *in vivo* half-life, an increased serum half-life or an increased AUC_{iv}.

Specific examples of such modifications are given further below. Moreover, the polypeptide variant of the invention may contain additional modifications giving rise to an enhanced phospholipid membrane binding affinity. Specific examples of such modifications are also given further below.

The term "variant" or "polypeptide variant" (of hFVII or hFVIIa) is intended to cover a polypeptide, which differs in one or more amino acid residues from SEQ ID NO:1, normally in 1-15 amino acid residues (for example in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues), such as in 1-10 amino acid residues, e.g. in 1-5 or 1-3 amino acid residues. In other words, a "variant" typically contains 1-15 amino acid modifications (for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid modifications), such as 1-10 amino acid modifications, e.g. 1-5 or 1-3 amino acid modifications relative to SEQ ID NO:1. In the present context, the term "modification" encompasses insertions, deletions, substitutions and combinations thereof. It will be understood that a polypeptide variant according to the present invention will be modified in at least one of the following positions: 196, 237 and/or 314.

The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

The term "polymerase chain reaction" or "PCR" generally refers to a method for amplification of a desired nucleotide sequence *in vitro*, as described, for example, in US 4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis, using oligonucleotide primers capable of hybridising preferentially to a template nucleic acid.

"Cell", "host cell", "cell line" and "cell culture" are used interchangeably herein and all such terms should be understood to include progeny resulting from growth or culturing of a cell.

"Transformation" and "transfection" are used interchangeably to refer to the process of introducing DNA into a cell.

5 "Operably linked" refers to the covalent joining of two or more nucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. For example, the nucleotide sequence encoding a presequence or secretory leader is operably linked to a nucleotide sequence coding for a polypeptide if it is expressed as a preprotein that participates in the secretion of the
10 polypeptide: a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the nucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction
15 sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

The terms "mutation" and "substitution" are used interchangeably herein.

In the context of the present invention the terms "modification" or "amino acid
20 modification" is intended to cover replacement of an amino acid side chain, substitution of an amino acid residue, deletion of an amino acid residue and insertion of an amino acid residue.

The term "introduce" refers to introduction of an amino acid residue, in particular by substitution of an existing amino acid residue, or alternatively by insertion of an additional amino acid residue.

The term "remove" refers to removal of an amino acid residue, in particular by
25 substitution of the amino acid residue to be removed by another amino acid residue, or alternatively by deletion (without substitution) of the amino acid residue to be removed.

The term "FVII" or "FVII polypeptide" refers to a FVII molecule provided in single chain form.

The term "FVIIa" or "FVIIa polypeptide" refers to a FVIIa molecule provided in its
30 activated two-chain form. When the amino acid sequence of SEQ ID NO:1 is used to describe

the amino acid sequence of FVIIa it will be understood that the peptide bond between R152 and I153 of the single-chain form has been cleaved, and that one of the chains comprises amino acid residues 1-152, the other chain amino acid residues 153-406.

The terms "rFVII" and "rFVIIa" refer to FVII and FVIIa polypeptides produced by
5 recombinant techniques.

The terms "hFVII" and "hFVIIa" refer to human wild-type FVII and FVIIa, respectively, having the amino acid sequence shown in SEQ ID NO:1

The terms "rhFVII" and "rhFVIIa" refer to human wild-type FVII and FVIIa, having the amino acid sequence shown in SEQ ID NO:1, produced by recombinant means. An example of
10 rhFVIIa is NovoSeven®.

The term "TF" means Tissue Factor.

The term "TFPI" means Tissue Factor Pathway Inhibitor.

The term "FX" means Factor X.

The term "Gla domain" is used about the first about 45 amino acid residues counted
15 from the N-terminus.

The term "protease domain" is used about residues 153-406 counted from the N-terminus.

The term "catalytic site" is used to mean the catalytic triad consisting of S344, D242 and H193 of the polypeptide variant.

20 The term "amidolytic activity" is intended to mean the activity measured in the "Amidolytic Assay" described herein. In order to exhibit "amidolytic activity" a variant of the invention, in its activated form, should have at least 10% of the amidolytic activity of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In a preferred embodiment of the invention the variant, in its activated form, has at least 20% of the amidolytic activity of rhFVIIa,
25 such as at least 30%, e.g. at least 40%, more preferably at least 50%, such as at least 60%, e.g. at least 70%, even more preferably at least 80%, such as at least 90% of the amidolytic activity of rhFVIIa when assayed in the "Amidolytic Assay" described herein. In an interesting embodiment the variant, in its activated form, has substantially the same amidolytic activity as rhFVIIa, such as an amidolytic activity of 75-125% of the amidolytic activity of rhFVIIa.

The term "clotting activity" is used to mean the activity measured in the "Whole Blood Assay" described herein. It will be understood that the activity measured in the "Whole Blood Assay" is the time needed to obtain clot formation. Thus, a lower clotting time corresponds to a higher clotting activity.

5 The term "increased clotting activity" is used to indicate that the clotting time of the polypeptide variant is statistically significantly decreased relative to that generated by rhFVIIa as determined under comparable conditions and when measured in the "Whole Blood Assay" described herein.

10 The term "immunogenicity" as used in connection with a given substance is intended to indicate the ability of the substance to induce a response from the immune system. The immune response may be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8th Edition, Blackwell) for further definition of immunogenicity). Normally, reduced antibody reactivity will be an indication of reduced immunogenicity. The reduced immunogenicity may be determined by use of any suitable method known in the art, e.g. *in vivo* or *in vitro*.

15 The term "functional *in vivo* half-life" is used in its normal meaning, i.e. the time at which 50% of the biological activity of the polypeptide variant is still present in the body/target organ, or the time at which the amidolytic or clotting activity of the polypeptide variant is 50% of the initial value.

20 As an alternative to determining functional *in vivo* half-life, "serum half-life" may be determined, i.e. the time at which 50% of the polypeptide variant circulates in the plasma or bloodstream prior to being cleared. Determination of serum half-life is often more simple than determining the functional *in vivo* half-life and the magnitude of serum half-life is usually a good indication of the magnitude of functional *in vivo* half-life. Alternatively terms to serum half-life include "plasma half-life", "circulating half-life", "serum clearance", "plasma clearance" and
25 "clearance half-life". The polypeptide variant is cleared by the action of one or more of the reticuloendothelial systems (RES), kidney, spleen or liver, by tissue factor, SEC receptor or other receptor mediated elimination, or by specific or unspecific proteolysis. Normally, clearance depends on size (relative to the cutoff for glomerular filtration), charge, attached carbohydrate chains, and the presence of cellular receptors for the protein. The functionality to be retained is

normally selected from procoagulant, proteolytic or receptor binding activity. The functional *in vivo* half-life and the serum half-life may be determined by any suitable method known in the art.

The term "increased" as used about the functional *in vivo* half-life or serum half-life is used to indicate that the relevant half-life of the polypeptide variant is statistically significantly increased relative to that of a reference molecule, such as a rhFVIIa as determined under comparable conditions (typically determined in an experimental animal, such as rats, rabbits, pigs or monkeys).

The term "AUC_{iv}" or "Area Under the Curve when administered intravenously" is used in its normal meaning, i.e. as the area under the activity in serum-time curve, where the polypeptide variant has been administered intravenously, in particular when administered intravenously in rats. Once the experimental activity-time points have been determined, the AUC_{iv} may conveniently be calculated by a computer program, such as GraphPad Prism 3.01.

The term "reduced sensitivity to proteolytic degradation" is primarily intended to mean that the polypeptide variant has reduced sensitivity to proteolytic degradation in comparison to rhFVIIa as determined under comparable conditions. Preferably, the proteolytic degradation is reduced by at least 10% (e.g. by 10-25% or by 10-50%), such as at least 25% (e.g. by 25-50%, by 25-75% or by 25-100%), more preferably by at least 35%, such as at least 50%, (e.g. by 50-75% or by 50-100%) even more preferably by at least 60%, such as by at least 75% (e.g. by 75-100%) or even at least 90%. Most preferably, the proteolytic degradation is reduced by at least 99%.

The term "renal clearance" is used in its normal meaning to indicate any clearance taking place by the kidneys, e.g. by glomerular filtration, tubular excretion or degradation in the tubular cells. Renal clearance depends on physical characteristics of the polypeptide, including size (diameter), hydrodynamic volume, symmetry, shape/rigidity, and charge. Normally, a molecular weight of about 67 kDa is considered to be a cut-off-value for renal clearance. Renal clearance may be established by any suitable assay, e.g. an established *in vivo* assay. Typically, renal clearance is determined by administering a labelled (e.g. radiolabelled or fluorescence labelled) polypeptide to a patient and measuring the label activity in urine collected from the patient. Reduced renal clearance is determined relative to a corresponding reference polypeptide, e.g. rh FVIIa, under comparable conditions. Preferably, the renal clearance rate of the

polypeptide variant is reduced by at least 50%, preferably by at least 75%, and most preferably by at least 90% compared to rhFVIIa.

Polypeptide variants of the invention

5 In its broadest aspect the present invention relates to a variant of FVII or FVIIa, wherein said variant comprises at least one modification in a position selected from the group consisting of 196, 237 and 341 as compared to hFVII or hFVIIa, preferably as compared to hFVIIa.

In the following sections preferred modifications in the above-mentioned positions are given.

10

Position 196

In one embodiment of the invention, the present invention relates to a variant of FVII or FVIIa, wherein said variant comprises at least one modification in position 196 as compared to hFVII or hFVIIa (SEQ ID NO:1).

15

In a preferred embodiment of the invention the modification in position 196 is a substitution, in particular D196N or D196K.

In a further interesting embodiment of the invention, the variant further comprises 1-15 amino acid modifications (e.g. substitutions), such as 1-10 amino acid modifications (e.g. substitutions), e.g. 1-5 amino acid modifications (e.g. substitutions) or 1-3 amino acid modifications (e.g. substitutions).

20

For example, the variant may contain at least one further amino acid modification made in the Gla domain as explained in the section entitled "Modifications in the Gla domain" below, and/or at least one further amino acid modification which leads to introduction of an *in vivo* N-glycosylation site as explained in the section entitled "Introduction of additional sugar moieties" below, and/or at least one further amino acid modification capable of increasing the intrinsic activity and/or at least one further amino acid modification which increases the TF-binding affinity. Examples of the latter modifications are described in the section entitled "Other modifications" below.

25

Position 237

In a further embodiment of the invention, the present invention relates to a variant of FVII or FVIIa, wherein said variant comprises at least one modification in position 237 as compared to hFVII or hFVIIa (SEQ ID NO:1).

5 In a preferred embodiment of the invention the modification in position 237 is a substitution, in particular G237L.

In a further interesting embodiment of the invention, the variant further comprises 1-15 amino acid modifications (e.g. substitutions), such as 1-10 amino acid modifications (e.g. substitutions), e.g. 1-5 amino acid modifications (e.g. substitutions) or 1-3 amino acid
10 modifications (e.g. substitutions).

For example, the variant may contain at least one further amino acid modification made in the Gla domain as explained in the section entitled "Modifications in the Gla domain" below, and/or at least one further amino acid modification which leads to introduction of an in vivo N-glycosylation site as explained in the section entitled "Introduction of additional sugar moieties"
15 below, and/or at least one further amino acid modification capable of increasing the intrinsic activity and/or at least one further amino acid modification which increases the TF-binding affinity. Examples of the latter modifications are described in the section entitled "Other modifications" below.

In still another embodiment of the invention the modification in position 237 is an
20 insertion. In an interesting embodiment the insertion is selected from the group consisting of G237GXX, G237GXXX and G237GXXXX, wherein X is any amino acid residue. Preferably, X is selected from the group consisting of Ala, Val, Leu, Ile, Gly, Ser and Thr, in particular Ala. Specific examples of preferred insertions include G237GAA, G237GAAA and G237GAAAA. Most preferably, the insertions are G237GAA.

25 In a further interesting embodiment of the invention, the variant further comprises 1-15 amino acid modifications (e.g. substitutions), such as 1-10 amino acid modifications (e.g. substitutions), e.g. 1-5 amino acid modifications (e.g. substitutions) or 1-3 amino acid modifications (e.g. substitutions).

For example, the variant may contain at least one further amino acid modification made
30 in the Gla domain as explained in the section entitled "Modifications in the Gla domain" below,

and/or at least one further amino acid modification which leads to introduction of an *in vivo* N-glycosylation site as explained in the section entitled "Introduction of additional sugar moieties" below, and/or at least one further amino acid modification capable of increasing the intrinsic activity and/or at least one further amino acid modification which increases the TF-binding affinity. Examples of the latter modifications are described in the section entitled "Other modifications" below.

Position 341

In a still further embodiment of the invention, the present invention relates to a variant of FVII or FVIIa, wherein said variant comprises at least one modification in position 341 as compared to hFVII or hFVIIa (SEQ ID NO:1).

In a preferred embodiment of the invention the modification in position 341 is a substitution, such as K341N or K341Q, in particular K341Q.

In a further interesting embodiment of the invention, the variant further comprises 1-15 amino acid modifications (e.g. substitutions), such as 1-10 amino acid modifications (e.g. substitutions), e.g. 1-5 amino acid modifications (e.g. substitutions) or 1-3 amino acid modifications (e.g. substitutions).

For example, the variant may contain at least one further amino acid modification made in the Gla domain as explained in the section entitled "Modifications in the Gla domain" below, and/or at least one further amino acid modification which leads to introduction of an *in vivo* N-glycosylation site as explained in the section entitled "Introduction of additional sugar moieties" below, and/or at least one further amino acid modification capable of increasing the intrinsic activity and/or at least one further amino acid modification which increases the TF-binding affinity. Examples of the latter modifications are described in the section entitled "Other modifications" below.

Properties of the variants of the invention

As explained previously, the variants disclosed herein have a decreased affinity for TFPI, which may be assessed using the BIAcore® Assays described herein. Using the BIAcore® assays it is possible to estimate various kinetic binding constants, such as the equilibrium dissociation constant, K_D , where $K_D = k_d/k_a$, where k_a is the association rate constant and k_d is the

dissociation rate constant. It will be understood that a higher value of K_D corresponds to a decreased affinity for TFPI.

Without being limited to any particular theory it is believed that k_a is controlled by diffusion. This means that when comparing the binding properties of hFVIIa or rhFVIIa with a variant of the invention, k_a may be assumed to be constant. Thus, decreased affinity for TFPI, giving rise to an increase in the K_D value, is mainly accounted for by an increase in k_d .

The variants of the invention possess an increased clotting activity (or a reduced clotting time) as compared to hFVIIa or rhFVIIa. In a preferred embodiment of the invention the ratio between the time to reach clot formation for the variant (t_{variant}) and the time to reach clot formation for hFVIIa or rhFVIIa (t_{wt}) is at the most 0.9 when assayed in the "Whole Blood Assay" described herein. More preferably the ratio ($t_{\text{variant}}/t_{\text{wt}}$) is at the most 0.75, such as 0.7, even more preferably the ratio ($t_{\text{variant}}/t_{\text{wt}}$) is at the most 0.6, most preferably the ratio ($t_{\text{variant}}/t_{\text{wt}}$) is at the most 0.5 when assayed in the "Whole Blood Assay" described herein.

15 Further modifications

As indicated above the FVII or FVIIa variant of the invention may comprise further modifications, in particular further modifications which confer additional advantageous properties to the FVII or FVIIa molecule. Thus, in addition to one or more of the modifications mentioned above, i.e. modifications in at least one position selected from the group consisting of 196, 237 and 341, the variant may comprise at least one further amino acid modification, in particular at least one further amino acid substitution.

In order to avoid too much disruption of the structure and function of the FVII or FVIIa polypeptide, the FVII or FVIIa polypeptide variant of the invention will typically have an amino acid sequence having more than 95% identity with SEQ ID NO:1, preferably more than 96% identity with SEQ ID NO:1, such as more than 97% identity with SEQ ID NO:1, more preferably at least 98% identity with SEQ ID NO:1, such as more than 99% identity with SEQ ID NO:1. Amino acid sequence homology/identity is conveniently determined from aligned sequences, using e.g. the ClustalW program, version 1.8, June 1999, using default parameters (Thompson et al., 1994, ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic

Acids Research, 22: 4673-4680) or from the PFAM families database version 4.0
(<http://pfam.wustl.edu/>) (*Nucleic Acids Res.* 1999 Jan 1; 27(1):260-2) by use of GENEDOC
version 2.5 (Nicholas, K.B., Nicholas H.B. Jr., and Deerfield, D.W. II. 1997 GeneDoc: Analysis
and Visualization of Genetic Variation, EMBNEW.NEWS 4:14; Nicholas, K.B. and Nicholas
5 H.B. Jr. 1997 GeneDoc: Analysis and Visualization of Genetic Variation).

Modifications in the Gla domain

In an interesting embodiment of the invention, at least one further amino acid
modification is made in the Gla domain, i.e. within the first about 45 amino acid residues
10 counted from the N-terminus of the FVII or FVIIa molecule. Preferably, no modifications are
made in residues 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35.

Without being limited by any particular theory, it is presently believed that an increased
clotting activity may be achieved by an enhanced binding affinity of the FVIIa molecule to the
phospholipid membranes present on the surface of activated platelets. This enhanced affinity is
15 believed to result in a higher local concentration of the activated FVIIa polypeptide in close
proximity to the other coagulation factors, particularly FX. Thus, the rate of activation of FX to
FXa will be higher, simply due to a higher molar ratio of the activated FVII polypeptide to FX.
The increased activation rate of FX then results in a higher amount of active thrombin, and thus a
higher rate of cross-linking of fibrin.

20 Thus, in a preferred embodiment according to this aspect of the invention, the
polypeptide variant has, in its activated form, an enhanced phospholipid membrane binding
affinity relative to the rhFVIIa polypeptide, in addition to its decreased affinity for TFPI.
Phospholipid membrane binding affinity may be measured by methods known in the art, such as
by the BIAcore® assays described in K. Nagata and H. Handa (Ads.), Real-Time Analysis of
25 Biomolecular Interactions, Springer-Verlag, Tokyo, 2000, Chapter 6 entitled "Lipid-Protein
Interactions".

A number of modifications in the FVII Gla domain leading to an increased membrane
binding affinity have been described in the art (see, for example, WO 99/20767 and WO
00/66753). Particular interesting positions in the Gla domain to be modified are positions P10,
30 K32, D33, A34 as well as insertion of an amino acid residue between A3 and F4. Thus, in a

preferred embodiment of the invention, the variant comprises, in addition to one or more of the modifications mentioned above a substitution in a position selected from the group consisting of P10, K32, D33 and A34 and combinations thereof as well as an insertion between A3 and F4. Particularly preferred positions are P10 and K32.

5 Preferably, the substitution to be made in position 32 is K32E, the substitution to be made in position 10 is P10Q, the substitution to be made in position 33 is D33F, the substitution to be made in position 34 is A34E and the insertion between A3 and F4 is preferably A3AY. In an interesting embodiment of the invention the variant comprises at least one of the following further modifications: A3AY, P10Q, K32E, D33F, A34E or combinations thereof. Most
10 preferably, the variant comprises one of the following further modifications: K32E, P10Q+K32E, A3AY+P10Q+K32E+D33F+A34E.

Introduction of non-polypeptide moieties

In another embodiment, the FVII or FVIIa variant has been further modified so that the
15 resulting polypeptide variant has increased functional *in vivo* half-life and/or increased plasma half-life and/or increased increased Area Under the Curve when administered intravenous (AUC_{iv}), in particular when administered intravenous in rats, and/or increased bioavailability and/or reduced sensitivity to proteolytic degradation. Consequently, medical treatment with a polypeptide variant according to this aspect of the invention offers a number of advantages over
20 the currently available rFVIIa compound, such as lower dosage and, optionally, longer duration between injections. Numerous examples of relevant amino acid substitutions are given in WO 01/58935.

The variants disclosed in WO 01/58935 are the result of a generally new strategy for developing improved FVII or FVIIa molecules. This strategy, in which non-polypeptide moieties
25 are attached to FVII/FVIIa variants, may also be used for the FVII or FVIIa variants of the present invention. More specifically, by removing and/or introducing an amino acid residue comprising an attachment group for a non-polypeptide moiety in the FVII or FVIIa polypeptide variant of the invention, it is possible to specifically adapt the polypeptide variant so as to make the molecule more susceptible to conjugation to a non-polypeptide moiety of choice, to optimize
30 the conjugation pattern (e.g. to ensure an optimal distribution and number of non-polypeptide

moieties on the surface of the FVII or FVIIa polypeptide variant and to ensure that only the attachment groups intended to be conjugated are present in the molecule) and thereby obtain a new conjugate molecule, which has activity and in addition one or more improved properties as compared to the FVII and FVIIa molecules available today. For instance, when the total number
5 of amino acid residues comprising an attachment group for the non-polypeptide of choice is increased or decreased to an optimized level, the renal clearance of the conjugate is typically significantly reduced due to the altered shape, size and/or charge of the molecule achieved by the conjugation.

Thus, interesting polypeptide variants according to this aspect of the present invention
10 are such polypeptides, wherein at least one amino acid residue comprising an attachment group for a non-polypeptide moiety has been introduced or removed in a FVII or FVIIa polypeptide variant comprising one or more of the TFPI-reducing modifications described above. Obviously, it is preferred that at least one non-polypeptide moiety is covalently attached to at least one of said attachment groups.

15 In interesting embodiments of the present invention more than one amino acid residue of the FVII or FVIIa polypeptide variant is altered, e.g. the alteration embraces removal as well as introduction of amino acid residues comprising an attachment group for the non-polypeptide moiety of choice. In addition to the removal and/or introduction of amino acid residues the polypeptide variant may comprise other substitutions or glycosylations that are not related to
20 introduction and/or removal of amino acid residues comprising an attachment group for the non-polypeptide moiety. Also, the polypeptide variant may be attached, e.g., to a serine proteinase inhibitor to inhibit the catalytic site of the polypeptide.

The amino acid residue comprising an attachment group for a non-polypeptide moiety, either it be removed or introduced, is selected on the basis of the nature of the non-polypeptide
25 moiety of choice and, in most instances, on the basis of the method in which conjugation between the polypeptide variant and the non-polypeptide moiety is to be achieved. For instance, when the non-polypeptide moiety is a polymer molecule such as a polyethylene glycol or polyalkylene oxide derived molecule, amino acid residues comprising an attachment group may be selected from the group consisting of lysine, cysteine, aspartic acid, glutamic acid, histidine,

and tyrosine, preferably lysine, cysteine, aspartic acid and glutamic acid, more preferably lysine and cysteine, in particular cysteine.

Whenever an attachment group for a non-polypeptide moiety is to be introduced into or removed from the FVII or FVIIa polypeptide variant, the position of the amino acid residue to be modified is preferably located at the surface of the FVII or FVIIa polypeptide, and more preferably occupied by an amino acid residue which has more than 25% of its side chain exposed to the surface (as defined in Example 1 herein), preferably more than 50% of its side chain exposed to the surface (as defined in Example 1 herein). Such positions have been identified on the basis of an analysis of a 3D structure of the hFVII or hFVIIa molecule as described in the Materials and Methods section herein.

Furthermore, the position is preferably selected from a part of the FVII molecule that is located outside the tissue factor binding site, the Gla domain, the active site region and/or the ridge of the active site binding cleft. These sites/regions are identified in Example 1 herein. It should be emphasized, however, that in certain situations, e.g. in case an inactivated polypeptide variant is desired, it may be advantageous to perform modifications in or close to the active site region and/or the ridge of the active site binding cleft. For example, it is contemplated that one or more attachment groups for the non-polypeptide moieties, such as attachment groups for *in vivo* N-glycosylation sites, may advantageously be introduced in the active site region or at the ridge of the active site binding cleft of the FVII variant.

In order to determine an optimal distribution of attachment groups, the distance between amino acid residues located at the surface of the FVII or FVIIa polypeptide variant is calculated on the basis of a 3D structure of the hFVII or hFVIIa polypeptide. More specifically, the distance between the CB's of the amino acid residues comprising such attachment groups, or the distance between the functional group (NZ for lysine, CG for aspartic acid, CD for glutamic acid, SG for cysteine) of one and the CB of another amino acid residue comprising an attachment group are determined. In case of glycine, CA is used instead of CB. In the FVII or FVIIa polypeptide variant of the invention, any of said distances is preferably more than 8 Å, in particular more than 10 Å in order to avoid or reduce heterogeneous conjugation.

In case of removal of an attachment group, the relevant amino acid residue comprising such group and occupying a position as defined above is preferably substituted with a different

amino acid residue that does not comprise an attachment group for the non-polypeptide moiety in question. Normally, the amino acid residue to be removed is one to which conjugation is disadvantageous, e.g. an amino acid residue located at or near a functional site of the polypeptide (since conjugation at such a site may result in inactivation or reduced activity of the resulting conjugate due to impaired receptor recognition). In the present context the term "functional site" is intended to indicate one or more amino acid residues which is/are essential for or otherwise involved in the function or performance of FVII or FVIIa. Such amino acid residues are a part of the functional site. The functional site may be determined by methods known in the art and is preferably identified by analysis of a structure of the FVIIa-TF complex (See Banner et al., Nature 1996; 380:41-46).

In case of introduction of an attachment group, an amino acid residue comprising such group is introduced into the position, preferably by substitution of the amino acid residue occupying such position.

The exact number of attachment groups present and available for conjugation in the FVII or FVIIa polypeptide variant is dependent on the effect desired to be achieved by the conjugation. The effect to be obtained is, e.g., dependent on the nature and degree of conjugation (e.g. the identity of the non-polypeptide moiety, the number of non-polypeptide moieties desirable or possible to conjugate to the polypeptide variant, where they should be conjugated or where conjugation should be avoided, etc.).

Functional *in vivo* half-life is *inter alia* dependent on the molecular weight of the protein, and the number of attachment groups needed for providing increased half-life thus depends on the molecular weight of the non-polypeptide moiety in question. In one embodiment, the polypeptide variant of the invention has a molecular weight of at least 67 kDa, in particular at least 70 kDa, e.g., as measured by SDS-PAGE according to Laemmli, U.K., Nature Vol 227 (1970), p680-85. FVII itself has a molecular weight of about 53 kDa, and therefore additional 10-20kDa is required to obtain the desired effect. This may, e.g., be provided by conjugating 2-4 10kDa PEG molecules or otherwise as described herein.

The polypeptide variant of the invention may contain 1-10 non-polypeptide moieties, typically 1-8 or 2-8 non-polypeptide moieties, preferably 1-5 or 2-5 non-polypeptide moieties,

such as 1-4 or 1-3 non-polypeptide moieties, e.g. 1, 2 or 3 non-polypeptide moieties, in particular PEG, such as mPEG or sugar moieties.

Introduction of additional sugar moieties

5 In an interesting embodiment of the invention the non-polypeptide moiety is a sugar moiety, i.e., the polypeptide variant of the invention is one which, in addition to one or more of the TFPI affinity-decreasing modifications comprises at least one sugar moiety covalently attached to an introduced glycosylation site. Preferably said glycosylation site is an *in vivo* glycosylation site, in particular an *in vivo* N-glycosylation site, which has been introduced by
10 substitution. Preferably, said glycosylation site is introduced in a position located outside the Gla domain, the tissue factor binding site, the active site region and the ridge of the active site binding cleft.

When used in the present context, the term "naturally occurring glycosylation site" covers the glycosylation sites at positions N145, N322, S52 and S60. In a similar way, the term
15 "naturally occurring *in vivo* O-glycosylation site" includes the positions S52 and S60, whereas the term "naturally occurring *in vivo* N-glycosylation site" includes positions N145 and N322.

Thus, in a very interesting embodiment of the invention, the non-polypeptide moiety is a sugar moiety and the introduced attachment group is a glycosylation site, preferably an *in vivo* glycosylation site, such as an *in vivo* O-glycosylation site or an *in vivo* N-glycosylation site, in
20 particular an *in vivo* N-glycosylation site. Typically, 1-10 glycosylation sites, in particular *in vivo* N-glycosylation sites, have been introduced, preferably 1-8, 1-6, 1-4 or 1-3 glycosylation sites. In particular, 1, 2 or 3 *in vivo* N-glycosylation sites have been introduced, preferably by substitution.

It will be understood that in order to prepare a polypeptide variant, wherein the FVII or
25 FVII polypeptide variant comprises one or more glycosylation sites, the polypeptide variant must be expressed in a host cell capable of attaching sugar (oligosaccharide) moieties at the glycosylation site(s). Examples of glycosylating host cells are given in the section further below entitled "Coupling to a sugar moiety".

Examples of positions, wherein the glycosylation sites may be introduced include, but
30 are not limited to, positions comprising an amino acid residue having an amino acid residue

having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), such as in a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein). The position is preferably selected from a part of the molecule that is located outside the TF binding site, the Gla domain, the active site region and the ridge of the active site cleft. These sites/regions are identified in Example 1 herein. It should be understood that when the term "at least 25% (or at least 50%) of its side chain exposed to the surface" is used in connection with introduction of an *in vivo* N-glycosylation site this term refers to the surface accessibility of the amino acid side chain in the position where the sugar moiety is actually attached. In many cases it will be necessary to introduce a serine or a threonine residue in position +2 relative to the asparagine residue to which the sugar moiety is actually attached (unless, of course, this position is already occupied by a serine or a threonine residue) and these positions, where the serine or threonine residues are introduced, are allowed to be buried, i.e. to have less than 25% (or 50%) of their side chains exposed to the surface.

Specific examples of such substitutions creating an *in vivo* N-glycosylation site include a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N, D334N and combinations thereof. In a preferred embodiment of the invention the *in vivo* N-glycosylation site is created by performing a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N, D334N and combinations thereof, more preferably by a substitution selected from the group consisting of T106N, A175T, I205T, V253N, T267N+S269T and combinations thereof, in particular I205T.

In one embodiment of the invention, one *in vivo* N-glycosylation site has been introduced by substitution. In another embodiment of the invention at least two *in vivo* N-glycosylation site sites, such as two *in vivo* N-glycosylation sites, have been introduced by substitution. Specific examples of substitutions creating two *in vivo* N-glycosylation sites include A51N+G58N, A51N+T106N, A51N+K109N, A51N+G124N, A51N+K143N+N145T, A51N+A175T, A51N+I205T, A51N+V253N, A51N+T267N+S269T, A51N+S314N+K316T,

A51N+R315N+V317T, A51N+K316N+G318T, A51N+G318N, A51N+D334N, G58N+T106N,
 G58N+K109N, G58N+G124N, G58N+K143N+N145T, G58N+A175T, G58N+I205T,
 G58N+V253N, G58N+T267N+S269T, G58N+S314N+K316T, G58N+R315N+V317T,
 G58N+K316N+G318T, G58N+G318N, G58N+D334N, T106N+K109N, T106N+G124N,
 5 T106N+K143N+N145T, T106N+A175T, T106N+I205T, T106N+V253N,
 T106N+T267N+S269T, T106N+S314N+K316T, T106N+R315N+V317T,
 T106N+K316N+G318T, T106N+G318N, T106N+D334N, K109N+G124N,
 K109N+K143N+N145T, K109N+A175T, K109N+I205T, K109N+V253N,
 K109N+T267N+S269T, K109N+S314N+K316T, K109N+R315N+V317T,
 10 K109N+K316N+G318T, K109N+G318N, K109N+D334N, G124N+K143N+N145T,
 G124N+A175T, G124N+I205T, G124N+V253N, G124N+T267N+S269T,
 G124N+S314N+K316T, G124N+R315N+V317T, G124N+K316N+G318T, G124N+G318N,
 G124N+D334N, K143N+N145T+A175T, K143N+N145T+I205T, K143N+N145T+V253N,
 K143N+N145T+T267N+S269T, K143N+N145T+S314N+K316T,
 15 K143N+N145T+R315N+V317T, K143N+N145T+K316N+G318T, K143N+N145T+G318N,
 K143N+N145T+D334N, A175T+I205T, A175T+V253N, A175T+T267N+S269T,
 A175T+S314N+K316T, A175T+R315N+V317T, A175T+K316N+G318T, A175T+G318N,
 A175T+D334N, I205T+V253N, I205T+T267N+S269T, I205T+S314N+K316T,
 I205T+R315N+V317T, I205T+K316N+G318T, I205T+G318N, I205T+D334N,
 20 V253N+T267N+S269T, V253N+S314N+K316T, V253N+R315N+V317T,
 V253N+K316N+G318T, V253N+G318N, V253N+D334N, T267N+S269T+S314N+K316T,
 T267N+S269T+R315N+V317T, T267N+S269T+K316N+G318T, T267N+S269T+G318N,
 T267N+S269T+D334N, S314N+K316T+R315N+V317T, S314N+K316T+G318N,
 S314N+K316T+D334N, R315N+V317T+K316N+G318T, R315N+V317T+G318N,
 25 R315N+V317T+D334N or G318N+D334N, preferably T106N+A175T, T106N+I205T,
 T106N+V253N, T106N+T267N+S269T, A175T+I205T, A175T+V253N,
 A175T+T267N+S269T, I205T+V253N, I205T+T267N+S269T or V253N+T267N+S269T, more
 preferably T106N+I205T, T106N+V253N or I205T+T267N+S269T.

In a still further embodiment of the invention at least three *in vivo* N-glycosylation site
 30 sites, such as three *in vivo* N-glycosylation sites, have been introduced by substitution. Specific

examples of substitutions creating three *in vivo* N-glycosylation sites include I205T+V253N+T267N+S269T and T106N+I205T+V253N.

In addition to a sugar moiety, the polypeptide variant according to the aspect of the invention described in the present section may contain additional non-polypeptide moieties; in particular a polymer molecule, as described in the present application, conjugated to one or more attachment groups present in the FVII or FVIIa variant.

It will be understood that any of the amino acid changes, in particular substitutions, specified in this section can be combined with any of the amino acid changes, in particular substitutions, specified in the other sections herein disclosing specific amino acid changes.

For instance, any of the glycosylated polypeptides variants disclosed in the present section having introduced and/or removed at least one glycosylation site may further be conjugated to a polymer molecule, such as PEG, or any other non-polypeptide moiety. For this purpose the conjugation may be achieved by use of attachment groups already present in the FVII or FVIIa polypeptide variant or attachment groups may have been introduced and/or removed, in particular such that a total of 1-6, such as 1, 2, 3, 4, 5, or 6 attachment groups are available for conjugation.

Introduction of non-polypeptide moieties that have cysteine as an attachment group

In a further interesting embodiment of the invention the non-polypeptide moiety has cysteine as an attachment group, i.e., the polypeptide variant of the invention is one which, in addition to one or more of the TFPI affinity-decreasing modifications described above comprises at least one non-polypeptide moiety covalently attached to an introduced cysteine. Preferably said cysteine residue is introduced by substitution. Preferably, said cysteine residue is introduced in a position located outside the TF binding site, the Gla domain, the active site region, and the ridge of the active site binding cleft.

FVII/FVIIa contains 22 cysteine residues located outside the Gla domain and disulfide bridges are established between the following cysteine residues: C50 and C61, C55 and C70, C72 and C81, C91 and C102, C98 and C112, C114 and C127, C135 and C262, C159 and C164, C178 and C194, C310 and C329, and between C340 and C368.

Thus, in an interesting embodiment of the invention at least one cysteine residue has been introduced, preferably by substitution, in the FVII or FVIIa polypeptide variant. Typically 1-10 cysteine residues have been introduced, preferably 1-8, 1-6, 1-4 or 1-3 cysteine residues have been introduced. In particular 1, 2 or 3 cysteine residues have been introduced, preferably
5 by substitution.

Examples of positions where the cysteine residues may be introduced include, but are not limited to, positions comprising an amino acid residue having an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein), such as in a position comprising an amino acid residue having at least 50% of its side chain exposed to the
10 surface (as defined in Example 1 herein). The position is preferably selected from a part of the molecule that is located outside the Gla domain, the active site region, the ridge of the active site binding cleft and the tissue factor-binding site. These sites/regions are identified in Example 1 herein.

In an interesting embodiment of the invention, a cysteine residue is introduced near or at
15 the C-terminus. For example, a cysteine residue may be introduced, either by substitution or insertion, in position 400-406. Specific examples of substitutions include: L400C, L401C, R402C, A403C, P404C, F405C and P406C, in particular P406C. Specific examples of insertions include L400LC, L401LC, R402RC, A403AC, P404PC, F405FC and P406PC, in particular P406PC.

20 While the non-polypeptide moiety according to this aspect of the invention may be any molecule which, when using the given conjugation method has cysteine as an attachment group it is preferred that the non-polypeptide moiety is a polymer molecule. The polymer molecule may be any of the molecules mentioned in the section entitled "Conjugation to a polymer molecule", but is preferably selected from the group consisting of linear or branched
25 polyethylene glycol or another polyalkylene oxide. In a particular interesting embodiment the polymer molecule is PEG, such as VS-PEG.

The conjugation between the polypeptide variant and the polymer may be achieved in any suitable manner, e.g. as described in the section entitled "Conjugation to a polymer molecule", e.g. in using a one step method or in the stepwise manner referred to in said section.

When the FVII or FVIIa polypeptide variant comprises only one conjugatable cysteine residue, this residue is preferably conjugated to a non-polypeptide moiety with a molecular weight of from about 5 kDa to about 20 kDa, e.g. from about 10 kDa to about 20 kDa, such as a molecular weight of about 5 kDa, about 10 kDa, about 12 kDa, about 15 kDa or about 20 kDa, either directly conjugated or indirectly through a low molecular weight polymer (as disclosed in WO 99/55377). When the FVII or FVIIa polypeptide variant comprises two or more conjugatable cysteine residues, normally each of the non-polypeptide moieties has a molecular weight of from about 5 to about 10 kDa, such as about 5 kDa or about 10 kDa.

It will be understood that any of the amino acid changes, in particular substitutions, specified in this section can be combined with any of the amino acid modifications specified in the other sections herein.

Other modifications

In a further embodiment of the present invention, the FVII or FVIIa variant may, in addition to the modifications described in the sections above, also contain mutations, which are already known to increase the intrinsic activity of the polypeptide, e.g. such as those described in WO 02/22776

Examples of preferred substitutions include substitutions selected from the group consisting of V158D, E296D, M298Q, L305V and K337A. More preferably, said substitutions are selected from the group consisting of V158D+E296D+M298Q+L305V+K337A, V158D+E296D+M298Q+K337A, V158D+E296D+M298Q+L305V, V158D+E296D+M298Q, M298Q, L305V+K337A, L305V and K337A.

Moreover, the variant may contain modifications which increase the TF binding affinity. Examples of such modifications include substitutions selected from the group consisting of L39E, L39Q, L39H, I42R, S43H, S43Q, K62E, K62R, L65Q, L65S, F71D, F71Y, F71E, F71Q, F71N, E82Q, E82N, E82K, F275H and combinations thereof, in particular L65Q, F71Y, K62E, S43Q and combinations thereof.

As already indicated above, the variant may also contain conservative amino acid substitutions.

The non-polypeptide moiety

As indicated further above the non-polypeptide moiety of the polypeptide variant of the invention is preferably selected from the group consisting of a polymer molecule, a lipophilic compound, a sugar moiety (by way of *in vivo* glycosylation) and an organic derivatizing agent.

- 5 All of these agents may confer desirable properties to the polypeptide variant, in particular increased functional *in vivo* half-life and/or increased plasma half-life.

The polypeptide variant is normally conjugated to only one type of non-polypeptide moiety, but may also be conjugated to two or more different types of non-polypeptide moieties, e.g. to a polymer molecule and a sugar moiety, to a lipophilic group and a sugar moiety, to an
10 organic derivatizing agent and a sugar moiety, to a lipophilic group and a polymer molecule, etc. The conjugation to two or more different non-polypeptide moieties may be done simultaneous or sequentially.

Methods of preparing a conjugated polypeptide variant of the invention

- 15 In the following sections "Conjugation to a lipophilic compound", "Conjugation to a polymer molecule", "Conjugation to a sugar moiety" and "Conjugation to an organic derivatizing agent" conjugation to specific types of non-polypeptide moieties is described. In general, a conjugated polypeptide variant according to the invention may be produced by culturing an appropriate host cell under conditions conducive for the expression of the
20 polypeptide, and recovering the polypeptide variant, wherein a) the polypeptide variant comprises at least one N- or O-glycosylation site and the host cell is a eukaryotic host cell capable of *in vivo* glycosylation, and/or b) the polypeptide variant is subjected to conjugation to a non-polypeptide moiety *in vitro*.

- It will be understood that the conjugation should be designed so as to produce the
25 optimal molecule with respect to the number of non-polypeptide moieties attached, the size and form of such molecules (e.g. whether they are linear or branched), and the attachment site(s) in the polypeptide variant. The molecular weight of the non-polypeptide moiety to be used may e.g. be chosen on the basis of the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugated polypeptide variant having a high molecular weight
30 (e.g. to reduce renal clearance) it is usually desirable to conjugate as few high molecular weight

non-polypeptide moieties as possible to obtain the desired molecular weight. When a high degree of shielding is desirable this may be obtained by use of a sufficiently high number of low molecular weight non-polypeptide moieties (e.g. with a molecular weight of from about 300 Da to about 5 kDa, such as a molecular weight of from 300 Da to 2 kDa) to effectively shield all or most protease cleavage sites or other vulnerable sites of the polypeptide variant.

Conjugation to a lipophilic compound

The polypeptide variant and the lipophilic compound may be conjugated to each other, either directly or by use of a linker. The lipophilic compound may be a natural compound such as a saturated or unsaturated fatty acid, a fatty acid diketone, a terpene, a prostaglandin, a vitamin, a carotenoide or steroide, or a synthetic compound such as a carbon acid, an alcohol, an amine and sulphonic acid with one or more alkyl-, aryl-, alkenyl- or other multiple unsaturated compounds. The conjugation between the polypeptide variant and the lipophilic compound, optionally through a linker may be done according to methods known in the art, e.g. as described by Bodanszky in *Peptide Synthesis*, John Wiley, New York, 1976 and in WO 96/12505.

Conjugation to a polymer molecule, including conjugation of a polymer molecule to the N-terminus of the polypeptide variant

The polymer molecule to be coupled to the polypeptide variant may be any suitable polymer molecule, such as a natural or synthetic homo-polymer or hetero-polymer, typically with a molecular weight in the range of about 300-100,000 Da, such as about 500-20,000 Da, more preferably in the range of about 500-15,000 Da, even more preferably in the range of about 2-12 kDa, such as in the range of about 3-10 kDa. When the term "about" is used herein in connection with a certain molecular weight, the word "about" indicates an approximate average molecular weight and reflects the fact that there will normally be a certain molecular weight distribution in a given polymer preparation.

Examples of homo-polymers include a polyol (i.e. poly-OH), a polyamine (i.e. poly-NH₂) and a polycarboxylic acid (i.e. poly-COOH). A hetero-polymer is a polymer comprising different coupling groups, such as a hydroxyl group and an amine group.

Examples of suitable polymer molecules include polymer molecules selected from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as polyethylene glycol (PEG) and polypropylene glycol (PPG), branched PEGs, poly-vinyl alcohol (PVA), poly-carboxylate, poly-(vinylpyrrolidone), polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextran, including carboxymethyl-dextran, or any other biopolymer suitable for reducing immunogenicity and/or increasing functional *in vivo* half-life and/or serum half-life. Another example of a polymer molecule is human albumin or another abundant plasma protein. Generally, polyalkylene glycol-derived polymers are biocompatible, non-toxic, non-antigenic, non-immunogenic, have various water solubility properties, and are easily excreted from living organisms.

PEG is the preferred polymer molecule, since it has only few reactive groups capable of cross-linking compared to, e.g., polysaccharides such as dextran. In particular, monofunctional PEG, e.g. methoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the polypeptide). Consequently, the risk of cross-linking is eliminated, the resulting conjugated polypeptide variants are more homogeneous and the reaction of the polymer molecules with the polypeptide variant is easier to control.

To effect covalent attachment of the polymer molecule(s) to the polypeptide variant, the hydroxyl end groups of the polymer molecule must be provided in an activated form, i.e. with reactive functional groups (examples of which include primary amino groups, hydrazide (HZ), thiol, succinate (SUC), succinimidyl succinate (SS), succinimidyl succinamide (SSA), succinimidyl propionate (SPA), succinimidyl carboxymethylate (SCM), benzotriazole carbonate (BTC), N-hydroxysuccinimide (NHS), aldehyde, nitrophenylcarbonate (NPC), and tresylate (TRES)). Suitable activated polymer molecules are commercially available, e.g. from Shearwater Polymers, Inc., Huntsville, AL, USA, or from PolyMASC Pharmaceuticals plc, UK.

Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g. as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Shearwater Polymers, Inc. 1997 and 2000 Catalogs (Functionalized Biocompatible Polymers for Research and pharmaceuticals, Polyethylene Glycol and Derivatives, incorporated herein by reference).

Specific examples of activated PEG polymers include the following linear PEGs: NHS-PEG (e.g. SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG, BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs such as PEG2-NHS and those disclosed in US 5,932,462 and US 5,643,575, both of which are incorporated herein by reference. Preferably, the above-mentioned activated linear PEG-polymers are in their mPEG forms. Furthermore, the following publications, incorporated herein by reference, disclose useful polymer molecules and/or PEGylation chemistries: US 5,824,778, US 5,476,653, WO 97/32607, EP 229,108, EP 402,378, US 4,902,502, US 5,281,698, US 5,122,614, US 5,219,564, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28024, WO 95/00162, WO 95/11924, WO 95/13090, WO 95/33490, WO 96/00080, WO 97/18832, WO 98/41562, WO 98/48837, WO 99/32134, WO 99/32139, WO 99/32140, WO 96/40791, WO 98/32466, WO 95/06058, EP 439 508, WO 97/03106, WO 96/21469, WO 95/13312, EP 921 131, US 5,736,625, WO 98/05363, EP 809 996, US 5,629,384, WO 96/41813, WO 96/07670, US 5,473,034, US 5,516,673, EP 605 963, US 5,382,657, EP 510 356, EP 400 472, EP 183 503 and EP 154 316.

The conjugation of the polypeptide variant and the activated polymer molecules is conducted by use of any conventional method, e.g. as described in the following references (which also describe suitable methods for activation of polymer molecules): R.F. Taylor, (1991), "Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.; S.S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Florida, USA; G.T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques", Academic Press, N.Y.). The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the polypeptide variant (examples of which are given further above), as well as the functional groups of the polymer (e.g. being amine, hydroxyl, carboxyl, aldehyde, sulfhydryl, succinimidyl, maleimide, vinylsulfone or haloacetate). The PEGylation may be directed towards conjugation to all available attachment groups on the polypeptide variant (i.e. such attachment groups that are exposed at the surface of the polypeptide) or may be directed towards one or more specific attachment groups, e.g. the N-

terminal amino group as described in US 5,985,265. Furthermore, the conjugation may be achieved in one step or in a stepwise manner (e.g. as described in WO 99/55377).

It will be understood that the PEGylation is designed so as to produce the optimal molecule with respect to the number of PEG molecules attached, the size and form of such molecules (e.g. whether they are linear or branched), and the attachment site(s) in the polypeptide variant. The molecular weight of the polymer to be used may e.g. be chosen on the basis of the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugated polypeptide variant having a high molecular weight (e.g. to reduce renal clearance) it is usually desirable to conjugate as few high molecular weight polymer molecules as possible to obtain the desired molecular weight. When a high degree of shielding is desirable this may be obtained by use of a sufficiently high number of low molecular weight polymer molecules (e.g. with a molecular weight of from about 300 Da to about 5 kDa) to effectively shield all or most protease cleavage sites or other vulnerable sites of the polypeptide. For instance, 2-8, such as 3-6 such polymers may be used.

In a particular interesting embodiment PEGylation is achieved by conjugating the PEG group(s) to introduced cysteine residues. Specific examples of activated PEG polymers particularly preferred for coupling to cysteine residues, include the following linear PEGs: vinylsulfone-PEG (VS-PEG), preferably vinylsulfone-mPEG (VS-mPEG); maleimide-PEG (MAL-PEG), preferably maleimide-mPEG (MAL-mPEG) and orthopyridyl-disulfide-PEG (OPSS-PEG), preferably orthopyridyl-disulfide-mPEG (OPSS-mPEG). Typically, such PEG or mPEG polymers will have a size of about 5 kDa, about 10 kDa, about 12 kDa or about 20 kDa.

In connection with conjugation to only a single attachment group on the polypeptide variant (e.g. the N-terminal amino group), it may be advantageous that the polymer molecule, which may be linear or branched, has a high molecular weight, preferably about 10-25 kDa, such as about 15-25 kDa, e.g. about 20 kDa.

Normally, the polymer conjugation is performed under conditions aimed at reacting as many of the available polymer attachment groups with polymer molecules. This is achieved by means of a suitable molar excess of the polymer relative to the polypeptide. Typically, the molar ratios of activated polymer molecules to polypeptide variant are up to about 1000-1, such as up

to about 200-1, or up to about 100-1. In some cases the ration may be somewhat lower, however, such as up to about 50-1, 10-1 or 5-1 in order to obtain optimal reaction.

It is also contemplated according to the invention to couple the polymer molecules to the polypeptide variant through a linker. Suitable linkers are well known to the skilled person. A preferred example is cyanuric chloride (Abuchowski et al., (1977), J. Biol. Chem., 252, 3578-3581; US 4,179,337; Shafer et al., (1986), J. Polym. Sci. Polym. Chem. Ed., 24, 375-378).

Subsequent to the conjugation, residual activated polymer molecules are blocked according to methods known in the art, e.g. by addition of primary amine to the reaction mixture, and the resulting inactivated polymer molecules are removed by a suitable method.

It will be understood that depending on the circumstances, e.g. the amino acid sequence of the polypeptide variant, the nature of the activated PEG compound being used and the specific PEGylation conditions, including the molar ratio of PEG to polypeptide, varying degrees of PEGylation may be obtained, with a higher degree of PEGylation generally being obtained with a higher ratio of PEG to polypeptide. The PEGylated polypeptides resulting from any given PEGylation process will, however, normally comprise a stochastic distribution of polypeptide conjugates having slightly different degrees of PEGylation.

One preferred method for selectively coupling polymer molecules, such as PEG molecules, to the N-terminus of the polypeptide variant is the method disclosed in US 5,985,265. This method involves reductive alkylation (reaction of the N-terminal amino group of the polypeptide variant with an aldehyde-containing polypeptide, such as aldehyde-PEG, in the presence of a reducing agent, such as NaCNBH_3). This method exploits differential reactivity of different types of primary amino groups (lysine versus N-terminal) available for derivatization in the polypeptide variant, thereby achieving substantially selective derivatization of the polypeptide variant at the N-terminus with a carbonyl group-containing polymer molecule, such as aldehyde-PEG. The reaction is performed at a pH which allows one to take advantage of the pK_a differences between the ϵ -amino groups of the lysine residues and that of the α -amino group of the N-terminal residue of the polypeptide. In order to achieve this differential reactivity, the reaction is typically carried at slightly acidic conditions. Specific examples of suitable pH ranges include pH 4.5-7, such as pH 4.5-6, e.g. pH 5-6, in particular about pH 5,

In another specific embodiment, the polypeptide variant of the invention comprises a PEG molecule attached to each of the lysine residues in the polypeptide available for PEGylation, in particular a linear or branched PEG molecule, e.g. with a molecular weight of about 1-15 kDa, typically about 2-12 kDa, such as about 3-10 kDa, e.g. about 5 or 6 kDa.

5 In yet another embodiment, the polypeptide variant of the invention comprises a PEG molecule attached to each of the lysine residues in the polypeptide available for PEGylation, and in addition to the N-terminal amino acid residue of the polypeptide variant.

Covalent *in vitro* coupling of carbohydrate moieties (such as dextran) to amino acid residues of the polypeptide variant may also be used, e.g. as described, for example in WO 10 87/05330 and in Aplin et al., CRC Crit Rev. Biochem, pp. 259-306, 1981. The *in vitro* coupling of carbohydrate moieties or PEG to protein- and peptide-bound Gln-residues can be carried out by transglutaminases (TGases). Transglutaminases catalyse the transfer of donor amine-groups to protein- and peptide-bound Gln-residues in a so-called cross-linking reaction. The donor-amine groups can be protein- or peptide-bound, such as the ϵ -amino-group in Lys-residues or it 15 can be part of a small or large organic molecule. An example of a small organic molecule functioning as amino-donor in TGase-catalysed cross-linking is putrescine (1,4-diaminobutane). An example of a larger organic molecule functioning as amino-donor in TGase-catalysed cross-linking is an amine-containing PEG (Sato et al., 1996, Biochemistry 35, 13072-13080).

TGases, in general, are highly specific enzymes, and not every Gln-residue exposed on 20 the surface of a protein is accessible to TGase-catalysed cross-linking to amino-containing substances. On the contrary, only few Gln-residues are naturally functioning as TGase substrates but the exact parameters governing which Gln-residues are good TGase substrates remain unknown. Thus, in order to render a protein susceptible to TGase-catalysed cross-linking reactions it is often a prerequisite at convenient positions to add stretches of amino acid sequence 25 known to function very well as TGase substrates. Several amino acid sequences are known to be or to contain excellent natural TGase substrates e.g. substance P, elafin, fibrinogen, fibronectin, α_2 -plasmin inhibitor, α -caseins, and β -caseins.

Coupling to a sugar moiety

In order to achieve *in vivo* glycosylation of the polypeptide variant of the invention, the nucleotide sequence encoding the polypeptide variant must be inserted in a glycosylating, eukaryotic expression host. The expression host cell may be selected from fungal (filamentous
5 fungal or yeast), insect or animal cells or from transgenic plant cells. In one embodiment the host cell is a mammalian cell, such as a CHO cell, a COS cell, a BHK cell or a HEK cell, e.g. a HEK 293 cell, or an insect cell, such as an SF9 cell, or a yeast cell, such as *S. cerevisiae* or *Pichia pastoris*, or any of the host cells mentioned hereinafter.

10 *Coupling to an organic derivatizing agent*

Covalent modification of the polypeptide variant may be performed by reacting one or more attachment groups of the polypeptide variant with an organic derivatizing agent. Suitable derivatizing agents and methods are well known in the art. For example, cysteinyl residues most commonly are reacted with α -haloacetates (and corresponding amines), such as chloroacetic acid
15 or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(4-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole. Histidyl residues are derivatized by reaction with
20 diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful. The reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0. Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α -amino-containing
25 residues include imidoesters such as methyl picolinimate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione and transaminase-catalyzed reaction with glyoxylate. Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction
30 be performed in alkaline conditions because of the high pKa of the guanidine functional group.

Furthermore, these reagents may react with the groups of lysine as well as the arginine guanidino group. Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides ($R-N=C=N-R'$), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Blocking of functional site

It has been reported that excessive polymer conjugation can lead to a loss of activity of a polypeptide to which the non-polypeptide moiety is conjugated. This problem can be eliminated, e.g., by removal of attachment groups located at the functional site or by reversible blocking the functional site prior to conjugation so that the functional site is blocked during conjugation. The latter strategy constitutes further embodiments of the invention (the first strategy being exemplified further above, e.g. by removal of lysine residues which may be located close to the functional site). More specifically, according to the second strategy the conjugation between the polypeptide variant and the non-polypeptide moiety is conducted under conditions where the functional site of the polypeptide variant is blocked by a helper molecule e.g. tissue factor capable of binding to the functional site of the polypeptide variant or a serine protease inhibitor.

Preferably, the helper molecule is one, which specifically recognizes a functional site of the polypeptide variant, such as a receptor, in particular tissue factor, either full length or a suitably truncated form of tissue factor or two molecules, one being tissue factor the other one being a peptide or peptide inhibitor binding to and thus protecting the area around the catalytic triad (preferably defined as amino acid residues within 10 Å of any atom in the catalytic triad).

Alternatively, the helper molecule may be an antibody, in particular a monoclonal antibody recognizing the polypeptide variant. In particular, the helper molecule may be a neutralizing monoclonal antibody.

The polypeptide variant is allowed to interact with the helper molecule before effecting conjugation. This ensures that the functional site of the polypeptide variant is shielded or protected and consequently unavailable for derivatization by the non-polypeptide moiety, such as

a polymer. Following its elution from the helper molecule, the conjugate between the non-polypeptide moiety and the polypeptide variant can be recovered with at least a partially preserved functional site.

The subsequent conjugation of the polypeptide variant having a blocked functional site to a polymer, a lipophilic compound, a sugar moiety, an organic derivatizing agent or any other compound is conducted in the normal way, e.g. as described in the sections above entitled "Conjugation to".

Irrespectively of the nature of the helper molecule to be used to shield the functional site of the polypeptide variant from conjugation, it is desirable that the helper molecule is free from or comprises only few attachment groups for the non-polypeptide moiety of choice in part(s) of the molecule, where the conjugation to such groups will hamper the desorption of the conjugated polypeptide variant from the helper molecule. Hereby, selective conjugation to attachment groups present in non-shielded parts of the polypeptide variant can be obtained and it is possible to reuse the helper molecule for repeated cycles of conjugation. For instance, if the non-polypeptide moiety is a polymer molecule, such as PEG, which has the epsilon amino group of a lysine or N-terminal amino acid residue as an attachment group, it is desirable that the helper molecule is substantially free from conjugatable epsilon amino groups, preferably free from any epsilon amino groups. Accordingly, in a preferred embodiment the helper molecule is a protein or peptide capable of binding to the functional site of the polypeptide, which protein or peptide is free from any conjugatable attachment groups for the non-polypeptide moiety of choice.

In a further embodiment the helper molecule is first covalently linked to a solid phase such as column packing materials, for instance Sephadex or agarose beads, or a surface, e.g. reaction vessel. Subsequently, the polypeptide variant is loaded onto the column material carrying the helper molecule and conjugation carried out according to methods known in the art, e.g. as described in the sections above entitled "Conjugation to". This procedure allows the conjugated polypeptide variant to be separated from the helper molecule by elution. The conjugated polypeptide variant is eluted by conventional techniques under physico-chemical conditions that do not lead to a substantive degradation of the conjugated polypeptide variant. The fluid phase containing the conjugated polypeptide variant is separated from the solid phase to which the helper molecule remains covalently linked. The separation can be achieved in other

ways: For instance, the helper molecule may be derivatised with a second molecule (e.g. biotin) that can be recognized by a specific binder (e.g. streptavidin). The specific binder may be linked to a solid phase thereby allowing the separation of the conjugated polypeptide variant from the helper molecule-second molecule complex through passage over a second helper-solid phase column which will retain, upon subsequent elution, the helper molecule-second molecule complex, but not the conjugated polypeptide variant. The conjugated polypeptide variant may be released from the helper molecule in any appropriate fashion. Deprotection may be achieved by providing conditions in which the helper molecule dissociates from the functional site of the polypeptide variant to which it is bound. For instance, a complex between an antibody to which a polymer is conjugated and an anti-idiotypic antibody can be dissociated by adjusting the pH to an acid or alkaline pH. Even more preferred is the use of a conformation specific antibody that recognizes a Ca^{2+} specific conformation of the polypeptide variant and consequently can be eluted with EDTA under mild conditions.

15 *Conjugation of a tagged polypeptide.*

In an alternative embodiment the polypeptide variant is expressed as a fusion protein with a tag, i.e. an amino acid sequence or peptide stretch made up of typically 1-30, such as 1-20 amino acid residues. Besides allowing for fast and easy purification, the tag is a convenient tool for achieving conjugation between the tagged polypeptide variant and the non-polypeptide moiety. In particular, the tag may be used for achieving conjugation in microtiter plates or other carriers, such as paramagnetic beads, to which the tagged polypeptide variant can be immobilised via the tag. The conjugation to the tagged polypeptide variant in, e.g., microtiter plates has the advantage that the tagged polypeptide variant can be immobilised in the microtiter plates directly from the culture broth (in principle without any purification) and subjected to conjugation. Thereby, the total number of process steps (from expression to conjugation) can be reduced. Furthermore, the tag may function as a spacer molecule, ensuring an improved accessibility to the immobilised polypeptide variant to be conjugated. The conjugation using a tagged polypeptide variant may be to any of the non-polypeptide moieties disclosed herein, e.g. to a polymer molecule such as PEG.

The identity of the specific tag to be used is not critical as long as the tag is capable of being expressed with the polypeptide variant and is capable of being immobilised on a suitable surface or carrier material. A number of suitable tags are commercially available, e.g. from Unizyme Laboratories, Denmark. For instance, the tag may consist of any of the following sequences:

His-His-His-His-His-His

Met-Lys-His-His-His-His-His

Met-Lys-His-His-Ala-His-His-Gln-His-His

Met-Lys-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln

10 Met-Lys-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln-Gln

or any of the following:

EQKLI SEEDL (a C-terminal tag described in Mol. Cell. Biol. 5:3610-16, 1985)

DYKDDDDK (a C- or N-terminal tag)

YPYDVDPDYA

15 Antibodies against the above tags are commercially available, e.g. from ADI, Aves Lab and Research Diagnostics.

The subsequent cleavage of the tag from the polypeptide variant may be achieved by use of commercially available enzymes.

20 **Methods of preparing a polypeptide variant of the invention**

The polypeptide variants of the present invention, optionally in glycosylated form, may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide variant and expressing the sequence in a suitable transformed or transfected host. Preferably, the host cell is a gammacarboxylating host cell such as a mammalian cell. However, polypeptide variants of the invention may be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

25 A nucleotide sequence encoding a polypeptide variant of the invention may be constructed by isolating or synthesizing a nucleotide sequence encoding hFVII and then

changing the nucleotide sequence so as to effect introduction (i.e. insertion or substitution) or removal (i.e. deletion or substitution) of the relevant amino acid residue(s).

The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence is prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide variant, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide variant will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide variant may be synthesized and assembled by PCR, ligation or ligation chain reaction (LCR) (Barany, PNAS 88:189-193, 1991). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

Alternative nucleotide sequence modification methods are available for producing polypeptide variants for high throughput screening, for instance methods which involve homologous cross-over such as disclosed in US 5,093,257, and methods which involve gene shuffling, i.e. recombination between two or more homologous nucleotide sequences resulting in new nucleotide sequences having a number of nucleotide alterations when compared to the starting nucleotide sequences. Gene shuffling (also known as DNA shuffling) involves one or more cycles of random fragmentation and reassembly of the nucleotide sequences, followed by screening to select nucleotide sequences encoding polypeptides with desired properties. In order for homology-based nucleic acid shuffling to take place, the relevant parts of the nucleotide sequences are preferably at least 50% identical, such as at least 60% identical, more preferably at least 70% identical, such as at least 80% identical. The recombination can be performed *in vitro* or *in vivo*.

Examples of suitable *in vitro* gene shuffling methods are disclosed by Stemmer et al. (1994), Proc. Natl. Acad. Sci. USA; vol. 91, pp. 10747-10751; Stemmer (1994), Nature, vol. 370, pp. 389-391; Smith (1994), Nature vol. 370, pp. 324-325; Zhao et al., Nat. Biotechnol. 1998, Mar; 16(3): 258-61; Zhao H. and Arnold, FB, Nucleic Acids Research, 1997, Vol. 25. No. 6 pp. 1307-1308; Shao et al., Nucleic Acids Research 1998, Jan 15; 26(2): pp. 681-83; and WO 95/17413.

An example of a suitable *in vivo* shuffling method is disclosed in WO 97/07205. Other techniques for mutagenesis of nucleic acid sequences by *in vitro* or *in vivo* recombination are disclosed e.g. in WO 97/20078 and US 5,837,458. Examples of specific shuffling techniques include "family shuffling", "synthetic shuffling" and "*in silico* shuffling".

5. Family shuffling involves subjecting a family of homologous genes from different species to one or more cycles of shuffling and subsequent screening or selection. Family shuffling techniques are disclosed e.g. by Crameri et al. (1998), Nature, vol. 391, pp. 288-291; Christians et al. (1999), Nature Biotechnology, vol. 17, pp. 259-264; Chang et al. (1999), Nature Biotechnology, vol. 17, pp. 793-797; and Ness et al. (1999), Nature Biotechnology, vol. 17, 893-
10 896.

Synthetic shuffling involves providing libraries of overlapping synthetic oligonucleotides based e.g. on a sequence alignment of homologous genes of interest. The synthetically generated oligonucleotides are recombined, and the resulting recombinant nucleic acid sequences are screened and if desired used for further shuffling cycles. Synthetic shuffling
15 techniques are disclosed in WO 00/42561.

In silico shuffling refers to a DNA shuffling procedure, which is performed or modelled using a computer system, thereby partly or entirely avoiding the need for physically manipulating nucleic acids. Techniques for *in silico* shuffling are disclosed in WO 00/42560.

Once assembled (by synthesis, site-directed mutagenesis or another method), the
20 nucleotide sequence encoding the polypeptide is inserted into a recombinant vector and operably linked to control sequences necessary for expression of the FVII in the desired transformed host cell.

It should of course be understood that not all vectors and expression control sequences function equally well to express the nucleotide sequence encoding the polypeptide variants
25 described herein. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in it or be able to integrate into the chromosome. The vector's copy number, the ability to control that copy number, and the
30 expression of any other proteins encoded by the vector, such as antibiotic markers, should also

be considered. In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the nucleotide sequence, their secretion characteristics, their ability to fold the polypeptide variant correctly, their fermentation or culture requirements, and the ease of purification of the products coded for by the nucleotide sequence.

The recombinant vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector is one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector, in which the nucleotide sequence encoding the polypeptide variant of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40; bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pCDNA3.1(+)\Hyg (Invitrogen, Carlsbad, CA, USA) and pCI-neo (Stratagene, La Jolla, CA, USA). Useful expression vectors for yeast cells include the 2 μ plasmid and derivatives thereof, the POT1 vector (US 4,931,373), the pJSO37 vector described in Okkels, Ann. New York Acad. Sci. 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate *et al.*, "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance And Expression of the Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986), pBluebac 4.5 and pMelbac (both available from Invitrogen). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host

range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages.

Other vectors for use in this invention include those that allow the nucleotide sequence encoding the polypeptide variant to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR
5 amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, "Construction Of A Modular Dihydrofolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) and glutamine synthetase ("GS") amplification (see, e.g., US 5,122,464 and EP 338,841).

10 The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2 μ replication genes REP 1-3 and origin of replication.

15 The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For *Saccharomyces*
20 *cerevisiae*, selectable markers include *ura3* and *leu2*. For filamentous fungi, selectable markers include *amdS*, *pyrG*, *arcB*, *niaD* and *sC*.

The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of the polypeptide variant of the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide
25 variant. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter.

A wide variety of expression control sequences may be used in the present invention.
30 Such useful expression control sequences include the expression control sequences associated

with structural genes of the foregoing expression vectors as well as any sequence known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, e.g. the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1 α (EF-1 α) promoter, the *Drosophila* minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus Elb region polyadenylation signals and the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug 20;196(4):947-50).

In order to improve expression in mammalian cells a synthetic intron may be inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, WI, USA).

Examples of suitable control sequences for directing transcription in insect cells include the polyhedrin promoter, the P10 promoter, the *Autographa californica* polyhedrosis virus basic protein promoter, the baculovirus immediate early gene 1 promoter and the baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence. Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast α -mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter, and the inducible GAL promoter.

Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger* α -amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidulans* acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator. Examples of suitable control sequences for use in bacterial host cells include promoters of the *lac* system, the *trp* system, the *TAC* or *TRC* system, and the major promoter regions of phage lambda.

The presence or absence of a signal peptide will, e.g., depend on the expression host cell used for the production of the polypeptide variant to be expressed (whether it is an intracellular or extracellular polypeptide) and whether it is desirable to obtain secretion. For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an

5. *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α -amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the *Lepidopteran manduca sexta* adipokinetic hormone precursor, (cf. US 5,023,328), the honeybee melittin (Invitrogen), ecdysteroid UDPglucosyltransferase (egt) (Murphy *et al.*, Protein Expression and Purification 4, 349-357 (1993) or human pancreatic lipase (hpl) (Methods in Enzymology 284, pp. 262-272, 1997). A preferred signal peptide for use in mammalian cells is that of hFVII or the murine Ig kappa light chain signal peptide (Coloma, M (1992) J. Imm. Methods 152:89-104). For use in yeast cells

15 suitable signal peptides have been found to be the α -factor signal peptide from *S. cerevisiae* (cf. US 4,870,008), a modified carboxypeptidase signal peptide (cf. L.A. Valls *et al.*, Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani *et al.*, Yeast 6, 1990, pp. 127-137), and the synthetic leader sequence TA57 (WO98/32867). For use in *E. coli* cells a suitable signal peptide have been

20 found to be the signal peptide *ompA* (EP 0 581 821).

The nucleotide sequence of the invention encoding a polypeptide variant, whether prepared by site-directed mutagenesis, synthesis, PCR or other methods, may optionally include a nucleotide sequence that encode a signal peptide. The signal peptide is present when the polypeptide variant is to be secreted from the cells in which it is expressed. Such signal peptide,

25 if present, should be one recognized by the cell chosen for expression of the polypeptide variant. The signal peptide will typically be the one normally associated with hFVII.

Any suitable host may be used to produce the polypeptide variant, including bacteria (although not particularly preferred), fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. Examples of

30 bacterial host cells include grampositive bacteria such as strains of *Bacillus*, e.g. *B. brevis* or *B.*

subtilis, or *Streptomyces*, or gramnegative bacteria, such as strains of *E. coli*. The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, e.g., Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278). Examples of suitable filamentous fungal host cells include strains of *Aspergillus*, e.g. *A. oryzae*, *A. niger*, or *A. nidulans*, *Fusarium* or *Trichoderma*. Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and US 5,679,543. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156 and WO 96/00787. Examples of suitable yeast host cells include strains of *Saccharomyces*, e.g. *S. cerevisiae*, *Schizosaccharomyces*, *Kluyveromyces*, *Pichia*, such as *P. pastoris* or *P. methanolica*, *Hansenula*, such as *H. Polymorpha* or *Yarrowia*. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153: 163; Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920; and as disclosed by Clontech Laboratories, Inc, Palo Alto, CA, USA (in the product protocol for the YeastmakerTM Yeast Transformation System Kit). Examples of suitable insect host cells include a *Lepidoptera* cell line, such as *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusia ni* cells (High Five) (US 5,077,214). Transformation of insect cells and production of heterologous polypeptides therein may be performed as described by Invitrogen. Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/O); Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC CRL-1573)), as well as plant cells in tissue culture. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville,

Maryland. Also, the mammalian cell, such as a CHO cell, may be modified to express sialyltransferase, e.g. 1,6-sialyltransferase, e.g. as described in US 5,047,335, in order to provide improved glycosylation of the polypeptide variant.

In order to increase secretion it may be of particular interest to produce the polypeptide variant of the invention together with an endoprotease, in particular a PACE (paired basic amino acid converting enzyme) (e.g. as described in US 5,986,079), such as a Kex2 endoprotease (e.g. as described in WO 00/28065).

Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection, liposome-mediated transfection, viral vectors and the transfection method described by Life Technologies Ltd, Paisley, UK using Lipofectamin 2000. These methods are well known in the art and e.g. described by Ausbel *et al.* (eds.), 1996, Current Protocols in Molecular Biology, John Wiley & Sons, New York, USA. The cultivation of mammalian cells are conducted according to established methods, e.g. as disclosed in Animal Cell Biotechnology, Methods and Protocols, Edited by Nigel Jenkins, 1999, Human Press Inc, Totowa, New Jersey, USA and Harrison MA and Rae IF, General Techniques of Cell Culture, Cambridge University Press 1997.

In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide variant using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide variant is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide variant is not secreted, it can be recovered from cell lysates.

The resulting polypeptide variant may be recovered by methods known in the art. For example, the polypeptide variant may be recovered from the nutrient medium by conventional

procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation.

The polypeptides may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), HPLC, or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

Single chain polypeptide variants of the invention can be purified and activated to two-chain polypeptide variants by a number of methods as described in the literature (Broze and Majerus, 1980, J. Biol. Chem. 255:1242-47 and Hedner and Kisiel, 1983, J.Clin.Invest. 71:1836-41). Another method whereby single chain polypeptide variants can be purified is by incorporation of Zn ions during purification as described in US 5,700,914.

In a preferred embodiment the polypeptide variant is purified as a single chain polypeptide variant, which further is optionally PEGylated. The optionally PEGylated single chain polypeptide variant is activated by either use of an immobilized enzyme (e.g. factors IIa, IXa, Xa and XIIa) or by autoactivation using a positively charged ion exchange matrix or the like.

It is advantageous to first purify the polypeptide variant in its single chain form, then PEGylate (if desired) and lastly activate by one of the methods described above or by autoactivation as described by Pedersen et al, 1989, Biochemistry 28: 9331-36. The advantage of carrying out PEGylation before activation is that PEGylation of the new amino-terminus formed by cleavage of R152-I153 is avoided. PEGylation of this new amino-terminus would render the molecule inactive since the formation of a hydrogen bond between D242 and the amino group of I153 is necessary for activity.

Pharmaceutical composition of the invention and its use

In a further aspect, the present invention relates to a composition, in particular to a pharmaceutical composition, comprising a polypeptide variant of the invention and a pharmaceutically acceptable carrier or excipient.

The polypeptide variant or the pharmaceutical composition according to the invention may be used as a medicament.

Due to the high clotting efficiency, the polypeptide variant of the invention, or the pharmaceutical composition of the invention, is particularly useful for the treatment of uncontrollable bleeding events in trauma patients, thrombocytopenic patients, patients in anticoagulant treatment, and cirrhosis patients with variceal bleeds, or other upper gastrointestinal bleedings, and in patients undergoing orthotopic liver transplantation, or liver resection (allowing for transfusion free surgery).

Trauma is defined as an injury to living tissue caused by an extrinsic agent. It is the 4th leading cause of death in the US and places a large financial burden on the economy.

Trauma is classified as either blunt or penetrative. Blunt trauma results in internal compression, organ damage and internal haemorrhage whereas penetrative trauma (as the consequence of an agent penetrating the body and destroying tissue, vessels and organs) results in external haemorrhage.

Haemorrhage, as a result of trauma, can start a cascade of problems. For example physiological compensation mechanisms are initiated with initial peripheral and mesenteric vasoconstriction to shunt blood to the central circulation. If circulation is not restored, hypovolemia shock (multiple organ failure due to inadequate perfusion) ensues. Since tissues throughout the body become starved for oxygen, anaerobic metabolism begins. However, the concomitant lactic acid leads the blood pH to drop and metabolic acidosis develops. If acidosis is severe and uncorrected, the patient may develop multisystem failure and die.

Although the majority of trauma patients are hypothermic on arrival in the emergency room due to the environmental conditions at the scene, inadequate protection, intravenous fluid administration and ongoing blood loss worsen the hypothermic state. Deficiencies in coagulation factors can result from blood loss or transfusions. Meanwhile, acidosis and hypothermia interfere with blood clotting mechanisms. Thus coagulopathy develops, which in turn, may mask surgical bleeding sites and hamper the control of mechanical bleeding. Hypothermia, coagulopathy and acidosis are often characterised as the "trauma triad of death"

Trauma may be caused by several events. For example, road traffic accidents result in many different types of trauma. Whilst some road traffic accidents are likely to result in

penetrative trauma, many road traffic accidents are likely to inflict blunt trauma to both head and body. However, these various types of trauma can all result in coagulopathy in the patient. Road traffic accidents are the leading cause of accidental death in the US. There are over 42,000 deaths from them in the US each year. Many trauma patients die at the location of the accident either
5 whilst being treated by the paramedics, before they arrive or in transit to the ER.

Another example includes gunshot wounds. Gunshot wounds are traumas that can result in massive bleeding. They are penetrative and destroy tissue as the bullet passes through the body, whether it be in the torso or a limb. In the US about 40,000 people a year die from gunshot wounds

10 A further example includes falls. Falls result in a similar profile of trauma type to road traffic accidents. By falling onto a solid object or the ground from height can cause both penetrative and decelerative blunt trauma. In the US, falls are a common cause of accidental death, numbering about 13,000.

A still further example includes machinery accidents. A smaller number of people die in
15 the US from machinery accident related deaths, whether struck by, or entangled in machinery. The figures are small but significant – around 2,000.

A still further example includes stab wounds. Stab wounds are penetrative injuries that can also cause massive bleeding. The organs most likely to be damaged in a stab wound are the liver, small intestine and the colon.

20 Cirrhosis of the liver is the terminal sequel of prolonged repeated injury to the hepatic parenchyma. The end result is the formation of broad bands of fibrous tissue separating regenerative nodules that do not maintain the normal organization of liver lobules and thus cause deteriorated liver function. Patients have prolonged prothrombin times as a result of the depletion of vitamin K-dependent coagulation factors. Pathogenetically, liver cirrhosis should be regarded
25 as the final common pathway of chronic liver injury, which can result from any form of intense repeated prolonged liver cell injury. Cirrhosis of the liver may be caused by direct liver injury, including chronic alcoholism, chronic viral hepatitis (types B, C, and D), and auto immune hepatitis as well as by indirect injury by way of bile duct damage, including primary biliary cirrhosis, primary sclerosing cholangitis and biliary atresia. Less common causes of cirrhosis

include direct liver injury from inherited disease such as cystic fibrosis, alpha-1-antitrypsin deficiency, hemochromatosis, Wilson's disease, galactosemia, and glycogen storage disease.

Transplantation is primarily reserved for late stage cirrhotic patients, where it is the key intervention for treating the disease. To be eligible for transplantation, a patient must be classified as Child's B or C, as well as meet additional criteria for selection. Last year, in the US alone, 4,954 transplants were performed.

It has been estimated that there are 6,000 bleeding episodes associated with patients undergoing resection each year. This correlates with the reserved position of this procedure although seems slightly high in comparison with transplantation numbers.

Accurate data on the incidence of variceal bleeding is hard to obtain. The key facts known are that at the time of diagnosis, varices are present in about 60% of decompensated and 30% of compensated patients and that about 30% of these patients with varices will experience a bleed and that each episode of variceal bleeding is associated with a 30% risk of mortality.

Thus, in a further aspect the present invention relates to a polypeptide variant of the invention for the manufacture of a medicament for the treatment of diseases or disorder wherein clot formation is desirable. A still further aspect of the present invention relates to a method for treating a mammal having a disease or disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of the polypeptide variant or the pharmaceutical composition of the invention.

Thrombocytopenia is caused by one of three mechanisms-decreased bone marrow production, increased splenic sequestration, or accelerated destruction of platelets.

Thrombocytopenia is a risk factor for hemorrhage, and platelet transfusion reduces the incidence of bleeding. The threshold for prophylactic platelet transfusion is 10,000/ μ l. In patients without fever or infections, a threshold of 5000/ μ l may be sufficient to prevent spontaneous hemorrhage. For invasive procedures, 50,000/ μ l platelets is the usual target level. In patients who develop antibodies to platelets following repeated transfusions, bleeding can be extremely difficult to control.

Examples of diseases/disorders wherein increased clot formation is desirable include, but is not limited to, hemorrhages, including brain hemorrhages, as well as patient with severe

uncontrolled bleedings, such as trauma. Further examples include patients undergoing living transplantations, patients undergoing resection and patients with variceal bleedings.

The polypeptide variant of the invention is administered to patients in a therapeutically effective dose, normally one approximately paralleling that employed in therapy with rhFVII such as NovoSeven®, or at lower dosage. By "therapeutically effective dose" herein is meant a dose that is sufficient to produce the desired effects in relation to the condition for which it is administered. The exact dose will depend on the circumstances, and will be ascertainable by one skilled in the art using known techniques. Normally, the dose should be capable of preventing or lessening the severity or spread of the condition or indication being treated. It will be apparent to those of skill in the art that an effective amount of a polypeptide variant or composition of the invention depends, *inter alia*, upon the disease, the dose, the administration schedule, whether the polypeptide variant or composition is administered alone or in conjunction with other therapeutic agents, the plasma half-life of the compositions, and the general health of the patient. Preferably, the polypeptide variant or composition of the invention is administered in an effective dose, in particular a dose which is sufficient to normalize the coagulation disorder.

The polypeptide variant of the invention is preferably administered in a composition including a pharmaceutically acceptable carrier or excipient. "Pharmaceutically acceptable" means a carrier or excipient that does not cause any untoward effects in patients to whom it is administered. Such pharmaceutically acceptable carriers and excipients are well known in the art (see, for example, Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company [1990]; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000]; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press [2000]).

The polypeptide variant of the invention can be formulated into pharmaceutical compositions by well-known methods. Suitable formulations are described by Remington's Pharmaceutical Sciences by E.W. Martin (Mark Publ. Co., 16th Ed., 1980).

The polypeptide variants of the invention can be used "as is" and/or in a salt form thereof. Suitable salts include, but are not limited to, salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium and magnesium, as well as e.g. zinc salts. These salts or complexes may be present as a crystalline and/or amorphous structure.

The pharmaceutical composition of the invention may be administered alone or in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical composition or may be administered separately from the polypeptide variant of the invention, either concurrently or in accordance with another treatment schedule. In addition, 5 the polypeptide variant or pharmaceutical composition of the invention may be used as an adjuvant to other therapies.

A "patient" for the purposes of the present invention includes both humans and other mammals. Thus, the methods are applicable to both human therapy and veterinary applications. The pharmaceutical composition comprising the polypeptide variant of the invention may be 10 formulated in a variety of forms, e.g. as a liquid, gel, lyophilized, or as a compressed solid. The preferred form will depend upon the particular indication being treated and will be apparent to one skilled in the art.

In particular, the pharmaceutical composition comprising the polypeptide variant of the invention may be formulated in lyophilised or stable soluble form. The polypeptide variant may 15 be lyophilised by a variety of procedures known in the art. The polypeptide variant may be in a stable soluble form by the removal or shielding of proteolytic degradation sites as described herein. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient and, in the case of emergencies, quicker action, which potentially can become life saving. The preferred form will depend upon the particular indication being treated and will be apparent 20 to one of skill in the art.

The administration of the formulations of the present invention can be performed in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, 25 vaginally, rectally, intraocularly, or in any other acceptable manner. The formulations can be administered continuously by infusion, although bolus injection is acceptable, using techniques well known in the art, such as pumps or implantation. In some instances the formulations may be directly applied as a solution or spray.

Parenterals

A preferred example of a pharmaceutical composition is a solution, in particular an aqueous solution, designed for parenteral administration. Although in many cases pharmaceutical solution formulations are provided in liquid form, appropriate for immediate use, such parenteral formulations may also be provided in frozen or in lyophilized form. In the former case, the composition must be thawed prior to use. The latter form is often used to enhance the stability of the active compound contained in the composition under a wider variety of storage conditions, as it is recognized by those skilled in the art that lyophilized preparations are generally more stable than their liquid counterparts. Such lyophilized preparations are reconstituted prior to use by the addition of one or more suitable pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution.

In case of parenterals, they are prepared for storage as lyophilized formulations or aqueous solutions by mixing, as appropriate, the polypeptide variant having the desired degree of purity with one or more pharmaceutically acceptable carriers, excipients or stabilizers typically employed in the art (all of which are termed "excipients"), for example buffering agents, stabilizing agents, preservatives, isotonifiers, non-ionic surfactants or detergents, antioxidants and/or other miscellaneous additives.

Buffering agents help to maintain the pH in the range which approximates physiological conditions. They are typically present at a concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents for use in the present invention include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium glyconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium

oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additional possibilities are phosphate buffers, histidine buffers and trimethylamine salts such as Tris.

5 Stabilizers refer to a broad category of excipients, which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, 10 etc., organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinositol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thiocetic acid, sodium thioglycolate, thioglycerol, α -monothioglycerol and sodium thiosulfate; low molecular weight polypeptides (i.e. <10 residues); proteins such as human serum 15 albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran. Stabilizers are typically present in the range of from 0.1 to 10,000 parts by weight based on the active protein weight.

20 Preservatives are added to retard microbial growth, and are typically added in amounts of about 0.2%-1% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides (e.g. benzalkonium chloride, bromide or iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, 25 cyclohexanol and 3-pentanol.

Isotonicifiers are added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Polyhydric alcohols can be present in an amount between 0.1% and 25% by weight, typically 1% to 5%, taking into account the relative 30 amounts of the other ingredients.

Non-ionic surfactants or detergents (also known as "wetting agents") may be present to help solubilizing the therapeutic agent as well as to protect the therapeutic polypeptide against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stress without causing denaturation of the polypeptide. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic® polyols, polyoxyethylene sorbitan monoethers (Tween®-20, Tween®-80, etc.).

Additional miscellaneous excipients include bulking agents or fillers (e.g. starch), chelating agents (e.g. EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E) and cosolvents.

10 The active ingredient may also be entrapped in microcapsules prepared, for example, by coascervation techniques or by interfacial polymerization, for example hydroxymethylcellulose, gelatin or poly-(methylmethacrylate) microcapsules, in colloidal drug delivery systems (for example liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences,
15 *supra*.

Parenteral formulations to be used for *in vivo* administration must be sterile. This is readily accomplished, for example, by filtration through sterile filtration membranes.

Sustained release preparations

20 Examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the polypeptide variant, the matrices having a suitable form such as a film or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) or poly(vinylalcohol)), polylactides, copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, 25 degradable lactic acid-glycolic acid copolymers such as the ProLease® technology or Lupron Depot® (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for long periods such as up to or over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated
30 polypeptides remain in the body for a long time, they may denature or aggregate as a result of

exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

The invention is further described in the following non-limiting examples.

10 MATERIALS AND METHODS

Accessible Surface Area (ASA)

The computer program Access (B. Lee and F.M.Richards, J. Mol.Biol. 55: 379-400 (1971)) version 2 (© 1983 Yale University) is used to compute the accessible surface area (ASA) of the individual atoms in the structure. This method typically uses a probe-size of 1.4Å and defines the Accessible Surface Area (ASA) as the area formed by the center of the probe. Prior to this calculation all water molecules and all hydrogen atoms should be removed from the coordinate set, as should other atoms not directly related to the protein.

20 *Fractional ASA of Side Chain*

The fractional ASA of the side chain atoms is computed by division of the sum of the ASA of the atoms in the side chain with a value representing the ASA of the side chain atoms of that residue type in an extended Ala-x-Ala tripeptide (See Hubbard, Campbell & Thornton (1991) J.Mol.Biol.220,507-530). For this example the CA atom is regarded as a part of the side chain of Glycine residues but not for the remaining residues. The following table is used as standard 100% ASA for the side chain:

Ala	69.23 Å ²		Leu	140.76 Å ²
Arg	200.35 Å ²		Lys	162.50 Å ²
Asn	106.25 Å ²		Met	156.08 Å ²

Asp	102.06 Å ²		Phe	163.90 Å ²
Cys	96.69 Å ²		Pro	119.65 Å ²
Gln	140.58 Å ²		Ser	78.16 Å ²
Glu	134.61 Å ²		Thr	101.67 Å ²
Gly	32.28 Å ²		Trp	210.89 Å ²
His	147.00 Å ²		Tyr	176.61 Å ²
Ile	137.91 Å ²		Val	114.14 Å ²

Residues not detected in the structure are defined as having 100% exposure as they are thought to reside in flexible regions. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all defined as being 100% exposed.

5

Determining Distances Between Atoms

The distance between atoms is most easily determined using molecular graphics software e.g. InsightII® v. 98.0, MSI INC.

10 *Active Site Region*

The active site region is defined as any residues having at least one atom within 10 Å of any atom in the catalytic triad (residues H193, D242, S344).

Determination of Tissue Factor Binding Site

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The TF binding site is defined as comprising all residues having their accessible surface area changed upon TF binding. This is determined by at least two ASA calculations; one on the isolated ligand(s) in the ligand(s)/receptor(s) complex and one on the complete ligand(s)/receptor(s) complex.

20 *Measurement of Reduced Sensitivity to Proteolytic Degradation*

Proteolytic degradation can be measured using the assay described in US 5,580,560, Example 5, where proteolysis is autoprotoleolysis.

Furthermore, reduced proteolysis can be tested in an *in vivo* model using radiolabelled samples and comparing proteolysis of rhFVIIa and the polypeptide variant of the invention by withdrawing blood samples and subjecting these to SDS-PAGE and autoradiography.

Irrespectively of the assay used for determining proteolytic degradation, "reduced proteolytic degradation" is intended to mean a measurable reduction in cleavage compared to that obtained by rhFVIIa as measured by gel scanning of Coomassie stained SDS-PAGE gels, HPLC or as measured by conserved catalytic activity in comparison to wild type using the tissue factor independent activity assay described below.

10 *Determination of the Molecular Weight of Polypeptide Variants*

The molecular weight of polypeptide variants is determined by either SDS-PAGE, gel filtration, Western Blots, matrix assisted laser desorption mass spectrometry or equilibrium centrifugation, e.g. SDS-PAGE according to Laemmli, U.K., Nature Vol 227 (1970), pp. 680-85.

15 *Determination of TFPI Inhibition*

FVII inhibition by TFPI can be monitored in the amidolytic assay described in Chang et al. Biochemistry 1999, 38: 10940-10948.

Determination of TFPI Affinity

20 The capacity of variants to bind to TFPI is evaluated using one or more of the three BIAcore® assays described in Dickinson et al. Proc. Natl. Acad. Sci. USA 1996, 93: 14379-14384; Roberge et al. Biochemistry 2001, 40: 9522-9531; and Ruf et al. Biochemistry 1999, 38(7): 1957-1966.

25 *TF-independent Factor X Activation Assay*

This assay has been described in detail on page 39826 in Nelsestuen et al., J Biol Chem, 2001; 276:39825-39831.

Briefly, the molecule to be assayed (either hFVIIa, rhFVIIa or the polypeptide variant of the invention in its activated form) is mixed with a source of phospholipid (preferably phosphatidylcholine and phosphatidylserine in a ratio of 8:2) and relipidated Factor X in Tris buffer

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containing BSA. After a specified incubation time the reaction is stopped by addition of excess EDTA. The concentration of factor Xa is then measured from absorbance change at 405 nm after addition of a chromogenic substrate (S-2222, Chromogenix). After correction from background the tissue factor independent activity of rhFVIIa (a_{wt}) is determined as the absorbance change after 10 minutes and the tissue factor independent activity of the polypeptide variant of the invention ($a_{variant}$) is also determined as the absorbance change after 10 minutes. The ratio between the activity of the polypeptide variant, in its activated form, and the activity of rhFVIIa is defined as $a_{variant}/a_{wt}$.

Clotting Assay

The clotting activity of the FVIIa and variants thereof were measured in one-stage assays and the clotting times were recorded on a Thrombotrack IV coagulometer (Medinor). Factor VII-depleted human plasma (American Diagnostica) was reconstituted and equilibrated at room temperature for 15-20 minutes. 50 microliters of plasma was then transferred to the coagulometer cups.

FVIIa and variants thereof were diluted in Glyoxaline Buffer (5.7 mM barbiturate, 4.3 mM sodium citrate, 117 mM NaCl, 1 mg/ml BSA, pH 7.35). The samples were added to the cup in 50 μ l and incubated at 37°C for 2 minutes.

Thromboplastin (Medinor) was reconstituted with water and $CaCl_2$ was added to a final concentration of 4.5 mM. The reaction was initiated by adding 100 μ l thromboplastin.

To measure the clotting activity in the absence of TF the same assay was used without addition of thromboplastin. Data was analysed using PRISM software.

Whole Blood Assay

The clotting activity of FVIIa and variants thereof were measured in one-stage assays and the clotting times were recorded on a Thrombotrack IV coagulometer (Medinor). 100 μ l of FVIIa or variants thereof were diluted in a buffer containing 10 mM glycylglycine, 50 mM NaCl, 37.5 mM $CaCl_2$, pH 7.35 and transferred to the reaction cup. The clotting reaction was initiated by addition of 50 μ l blood containing 10% 0.13 M tri-sodium citrate as anticoagulant. Data was analysed using Excel or PRISM software.

Amidolytic Assay

The ability of the variants to cleave small peptide substrates can be measured using the chromogenic substrate S-2288 (D-Ile-Pro-Arg-p-nitroanilide). FVIIa is diluted to about 10-90 nM in assay buffer (50 mM Na-Hepes pH 7.5, 150 mM NaCl, 5 mM CaCl₂, 0.1% BSA, 1U/ml Heparin). Furthermore, soluble TF (sTF) is diluted to 50-450 nM in assay buffer. 120 µl of assay buffer is mixed with 20 µl of the FVIIa sample and 20 µl sTF. After 5 min incubation at room temperature with gentle shaking, followed by 10 min incubation at 37°C, the reaction is started by addition of the S-2288 substrate to 1 mM and the absorption at 405 nm is determined at several time points.

10

ELISA Assay

FVII/FVIIa (or variant) concentrations are determined by ELISA. Wells of a microtiter plate are coated with an antibody directed against the protease domain using a solution of 2 µg/ml in PBS (100 µl per well). After overnight coating at R.T., the wells are washed 4 times with THT buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.2 0.05% Tween-20). Subsequently, 200 µl of 1% Casein (diluted from 2.5% stock using 100 mM NaCl, 50 mM Tris-HCl pH 7.2) is added per well for blocking. After 1 hr incubation at R.T., the wells are emptied, and 100 µl of sample (optionally diluted in dilution buffer (THT + 0.1% Casein)) is added. After another incubation of 1 hr at room temperature, the wells are washed 4 times with THT buffer, and 100 µl of a biotin-labelled antibody directed against the EGF-like domain (1 µg/ml) is added. After another 1 hr incubation at R.T., followed by 4 more washes with THT buffer, 100 µl of streptavidin-horse radish peroxidase (DAKO A/S, Glostrup, Denmark, 1/10000 diluted) is added. After another 1 hr incubation at R.T., followed by 4 more washes with THT buffer, 100 µl of TMB (3,3',5,5'-tetramethylbenzidine, Kem-en-Tech A/S, Denmark) is added. After 30 min incubation at R.T. in the dark, 100 µl of 1 M H₂SO₄ is added and OD_{450nm} is determined. A standard curve is prepared using rhFVIIa (NovoSeven®).

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Alternatively, FVII/FVIIa or variants may be quantified through the Gla domain rather than through the protease domain. In this ELISA set-up, wells are coated overnight with an antibody directed against the EGF-like domain and for detection, a calcium-dependent biotin-

labelled monoclonal anti-Gla domain antibody is used (2 µg/ml, 100 µl per well). In this set-up, 5 mM CaCl₂ is added to the THT and dilution buffers.

EXAMPLES

Example 1

The X-ray structure of hFVIIa in complex with soluble tissue factor by Banner et al., J Mol Biol, 1996; 285:2089 is used for this example. It is noted that the numbering of residues in the reference does not follow the sequence. Here, we have used the sequential numbering according to SEQ ID NO:1. The gamma-carboxy glutamic acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 are all here named Glu (three letter abbreviation) or E (one letter abbreviation). Residues 143-152 are not present in the structure.

Surface Exposure

Performing fractional ASA calculations on FVII fragments alone combined with the definition of accessibilities of non standard and/or missing residues described in the methods resulted in the following residues having more than 25% of their side chain exposed to the surface: A1, N2, A3, F4, L5, E6, E7, L8, R9, P10, S12, L13, E14, E16, K18, E19, E20, Q21, S23, F24, E25, E26, R28, E29, F31, K32, D33, A34, E35, R36, K38, L39, W41, I42, S43, S45, G47, D48, Q49, A51, S52, S53, Q56, G58, S60, K62, D63, Q64, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, T83, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, E99, S103, D104, H105, T106, G107, T108, K109, S111, R113, E116, G117, S119, L120, L121, A122, D123, G124, V125, S126, T128, P129, T130, V131, E132, I140, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, V158, P160, K161, E163, L171, N173, G174, A175, N184, T185, I186, H193, K197, K199, N200, R202, N203, I205, S214, E215, H216, D217, G218, D219, S222, R224, S232, T233, V235, P236, G237, T238, T239, N240, H249, Q250, P251, V253, T255, D256, E265, R266, T267, E270, R271, F275, V276, R277, F278, L280, L287, L288, D289, R290, G291, A292, T293, L295, E296, N301, M306, T307, Q308, D309, L311, Q312, Q313, R315, K316, V317, G318, D319, S320, P321, N322, T324, E325, Y326, Y332, S333, D334, S336, K337, K341, G342, H351, R353,

G354, Q366, G367, T370, V371, G372, R379, E385, Q388, K389, R392, S393, E394, P395, R396, P397, G398, V399, L400, L401, R402, P404 and P406.

The following residues had more than 50% of their side chain exposed to the surface:

A1, A3, F4, L5, E6, E7, L8, R9, P10, E14, E16, K18, E19, E20, Q21, S23, E25, E26, E29, K32,
5 A34, E35, R36, K38, L39, I42, S43, G47, D48, A51, S52, S53, Q56, G58, S60, K62, L65, Q66,
S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, H84, K85, D86, D87, Q88, L89, I90, V92,
N93, E94, G97, T106, G107, T108, K109, S111, E116, S119, L121, A122, D123, G124, V131,
E132, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155,
K157, P160, N173, G174, A175, K197, K199, N200, R202, S214, E215, H216, G218, R224,
10 V235, P236, G237, T238, H249, Q250, V253, D256, T267, F275, R277, F278, L288, D289,
R290, G291, A292, T293, L295, N301, M306, Q308, D309, L311, Q312, Q313, R315, K316,
G318, D319, N322, E325, D334, K341, G354, G367, V371, E385, K389, R392, E394, R396,
P397, G398, R402, P404 and P406.

15 *Tissue Factor Binding Site*

Performing ASA calculations the following residues in hFVII change their ASA in the complex. These residues were defined as constituting the tissue factor binding site: L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277,
20 F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379.

Active Site Region

The active site region is defined as any residue having at least one atom within a distance of 10 Å from any atom in the catalytic triad (residues H193, D242, S344): I153, Q167,
25 V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191,
A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234,
V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282,
G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341,
G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, I361, V362, S363, W364,
30 G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405.

The Ridge of the Active Site Binding Cleft

The ridge of the active site binding cleft region was defined by visual inspection of the FVIIa structure 1FAK.pdb as: N173, A175, K199, N200, N203, D289, R290, G291, A292, P321 and T370.

Example 2

Design of an expression cassette for expression of hFVII in mammalian cells

The DNA sequence shown in SEQ ID NO:2, encompassing the short form of the full length cDNA encoding hFVII with its native short signal peptide (Hagen et al., 1986. PNAS 83:2412), was synthesized in order to facilitate high expression in mammalian cells. First the ATG start codon context was modified according to the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug 20;196(4):947-50), so that there is a perfect match to the consensus sequence upstream of the ATG start codon. Secondly the open reading frame of the native cDNA was modified by making a bias in the codon usage towards the codons frequently used in highly expressed human genes. Further, two translational stop codons were inserted at the end of the open reading frame in order to facilitate efficient translational stop. The fully synthetic and expression optimized hFVII gene was assembled from 70-mer DNA oligonucleotides and finally amplified using end primers inserting *Bam*HI and *Hind*III sites at the 5' and 3' ends respectively using standard PCR techniques, which resulted in the following sequence:

ggatcccgccaccatggtcagccaggccctccgcctcctgtgcctgctcctggggctgcagggctgcctggctgccgtcttcgtcaccag
gaggaagcccatggcgtcctgcatcgccggcgccggccaatgcctttctggaagagctccgccctggctccctggaacgcgaatgcaa
agaggaacagtgcagctttgaggaagccgggagatttcaaagacgctgagcggacaaaactgtttggattagctatagcgtatggcgat
cagtgcctccagccctgccagaacgggggctcctgcaaagaccagctgcagagctatatctgcttctgcctgccttgaggggc
gcaattgcgaaccataaggatgaccagctgatttcgtcaacgaaaacgggggctgcgagcagtactgcagcgatcacacgggcacg
aagcggagctgccgctgccacgaaggctatagcctcctggctgacgggggtcctgcacgccacgggtggaatacccttgcgggaagatt
ccattctagaaaagcgggaacgctagcaaacccagggccggatcgtcgcggaaggtctgccctaagggggagtgccctggcagg
tcctgctcctggtaacggggccagctgtgcggcgggaccctcatcaataccattgggtcgtgtcccgctcactgcttcgataagatta
agaattggcggaaacctatcgtgtgctcggcgaacacgatctgtccgagcatgacggggacgaacagtcgccggggtggctcaggtc
atcattccctccacctatgtgcctggcacgaccaatcacgatatcgctctgctccgcctccaccagcccgtgtgtcaccgatcacgtcgtg

cctctgtgctgcctgagcggaccttagcgaacgcacgctggcttgcgcttagcctcgtgtccggctggggccagctgctcgaccg
 gggcgctaccgctctcgagctgatgggtgctcaacgtccccggctgatgaccaggactgcctgcagcagctccgcaaagtgggggact
 cccccaatacacggagtatatgtttgctgctggtatagcgtggctccaaggatagctgcaagggggactccggcggggcccatgccac
 gcactatcgcgggacctggtacctcaccgggatcgtcagctggggccagggtgcgccacgggtggggcactttggcgtctacacgcgcg
 5 tcagccagtacattgagtggctgcagaagctcatgcggagcgaaccccgcccggggtgctcctgcggggccctttcccttgataaaagct
 t

A vector for the cloning of the generated PCR product encompassing the expression
 cassette for hFVII was prepared by cloning the intron from pCINeo (Promega). The synthetic
 10 intron from pCI-Neo was amplified using standard PCR conditions and the primers:

CBProFpr174: 5'- AGCTGGCTAGCCACTGGGCAGGTAAGTATCA -3' and

CBProFpr175: 5'- TGGCGGGATCCTTAAGAGCTGTAATTGAACT -3'

15 resulting in a 332 bp PCR fragment. The fragment was cut with *NheI* and *BamHI* before cloning
 into pCDNA3.1/HygR (obtained from Invitrogen) resulting in PF#34.

The expression cassette for hFVII was cloned between the *BamHI* and *HindIII* sites of
 PF#34, resulting in plasmid PF#226.

20 **Example 3**

Construction of expression vectors encoding polypeptide variants of the invention

Sequence overhang extension (SOE) PCR was used for generating constructs having
 variant FVII open reading frames with substituted codons. In the SOE-PCR both the N-terminal
 part and the C-terminal part of the FVII open reading frame was first amplified in individual
 25 primary PCRs.

In order to change the codon for D196 to the codon for N196 the following primers
 were used pair wise for the primary PCRs:

CB499: 5'- CCCATTCTAGAAAAGCGGAACGCCAGCAAACCCCAGGG -3' and

30 CB562: 5'- CCAATTCTTAATCTTGTGGAAGCAGTGAGCGGCG -3',

and

CB256: 5'- CTCCGTGATATTGGGGGAGTC -3' and

CB561: 5'- CGCCGCTCACTGCTTCAACAAGATTAAGAATTGG -3'.

5 The primary PCR products were then combined and the terminal primers (CB499 and CB256) added allowing for the secondary full-length product encoding the mutated fragment of the desired D196N variant to be made. This PCR product was restricted with *Xba*I and *Xho*I and used to substitute the equivalent fragment of the FVII coding region of expression vector PF226 resulting in the expression vector pB0014 encoding the D196N variant.

10 With the exception of the constructs for position 341 variants, the constructs were made in the same way as for D196N. Constructs for position 341 variants were made using the end primers

CB220: 5'- CGCTCTCGAGCTGATGGTGCTC - 3' and

15 CB362: 5'- CAAACAACAGATGGCTGGCAAC - 3'

allowing for directional cloning between *Xho*I and *Hind*III. The central primer used in the SOE-PCR reactions for the substitution variants were:

20 D196K

CB563: CGCCGCTCACTGCTTCAAGAAGATTAAGAATTGG

CB564: CCAATTCTTAATCTTCTTGAAGCAGTGAGCGGCG

G237L

25 CB565: CTCCACCTATGTGCCTCTGACGACCAATCACGA

CB566: TCGTGATTGGTCGTCAGAGGCACATAGGTGGAG

K341Q

CB569: CCAAGGATGCCAGGGGGGACTCCGGCGGGC

30 CB570: GCCCGCCGGAGTCCCCCTGGCAGCTATCCTTGG

For the insertion variant the central primers was:

G237GAA

CB597: ACCTATGTGCCTGGCGCTGCCACGACCAATCACGAT

CB598: ATCGTGATTGGTCGTGGCAGCGCCAGGCACATAGGT

Example 4

Expression of FVII or FVII variants in CHO K1 cells

The cell line CHO K1 (ATCC # CCL-61) is seeded at 50% confluence in T-25 flasks using MEM α , 10% FCS (Gibco/BRL Cat # 10091), P/S and 5 μ g/ml phyloquinone and allowed to grow until confluent. The confluent mono cell layer is transfected with 5 μ g of the relevant plasmid described above using the Lipofectamine 2000 transfection agent (Life technologies) according to the manufacturer's instructions. Twenty four hours post transfection a sample is drawn and quantified using e.g. an ELISA recognizing the EGF1 domain of hFVII. At this time point relevant selection (e.g. Hygromycin B) may be applied to the cells with the purpose of generating a pool of stable transfectants. When using CHO K1 cells and the Hygromycin B resistance gene as selectable marker on the plasmid, this is usually achieved within one week.

Example 5

Generation of CHO-K1 cells stably expressing polypeptide variants

A vial of CHO-K1 transfectant pool is thawed and the cells seeded in a 175 cm² tissue flask containing 25 ml of MEM α , 10% FCS, phyloquinone (5 μ g/ml), 100 U/l penicillin, 100 μ g/l streptomycin and grown for 24 hours. The cells are harvested, diluted and plated in 96 well microtiter plates at a cell density of 1/2-1 cell/well. After a week of growth, colonies of 20-100 cells are present in the wells and those wells containing only one colony are labelled. After a further two weeks, the media in all wells containing only one colony is substituted with 200 μ l fresh medium. After 24 hours, a medium sample is withdrawn and analysed by e.g. ELISA. High producing clones are selected and used to produce FVII or variant on large scale.

Example 6*Purification of polypeptide variants and subsequent activation*

FVII and FVII variants are purified as follows: The procedure is performed at 4°C. The harvested culture media from large-scale production is ultrafiltered using a Millipore TFF system with 30 KDa cut-off Pellicon membranes. After concentration of the medium, citrate is added to 5 mM and the pH is adjusted to 8.6. If necessary, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose FF column, equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6. After washing the column with 100 mM NaCl, 10 mM Tris pH 8.6, followed by 150 mM NaCl, 10 mM Tris pH 8.6, FVII is eluted using 10 mM Tris, 25 mM NaCl, 35 mM CaCl₂, pH 8.6.

For the second chromatographic step, an affinity column is prepared by coupling of a monoclonal Calcium-dependent antiGla-domain antibody to CNBr-activated Sepharose FF. About 5.5 mg antibody is coupled per ml resin. The column is equilibrated with 10 mM Tris, 100 mM NaCl, 35 mM CaCl₂, pH 7.5. NaCl is added to the sample to a concentration of 100 mM NaCl and the pH is adjusted to 7.4 -7.6. After O/N application of the sample, the column is washed with 100 mM NaCl, 35 mM CaCl₂, 10 mM Tris pH 7.5, and the FVII protein is eluted with 100 mM NaCl, 50 mM citrate, 75 mM Tris pH 7.5.

For the third chromatographic, the conductivity of the sample is lowered to below 10 mS/cm, if necessary, and the pH is adjusted to 8.6. The sample is then applied to a Q-sepharose column (equilibrated with 50 mM NaCl, 10 mM Tris pH 8.6) at a density around 3-5 mg protein per ml gel to obtain efficient activation. After application, the column is washed with 50 mM NaCl, 10 mM Tris pH 8.6 for about 4 hours with a flow of 3-4 column volumes (cv) per hour. The FVII protein is eluted using a gradient of 0-100% of 500 mM NaCl, 10 mM Tris pH 8.6 over 40 cv. FVII containing fractions are pooled.

For the final chromatographic step, the conductivity is lowered to below 10 mS/cm. Subsequently, the sample is applied to a Q-sepharose column (equilibrated with 140 mM NaCl, 10 mM glycylglycine pH 8.6) at a concentration of 3-5 mg protein per ml gel. The column is then washed with 140 mM NaCl, 10 mM glycylglycine pH 8.6 and FVII is eluted with 140 mM NaCl, 15 mM CaCl₂, 10 mM glycylglycine pH 8.6. The eluate is diluted to 10 mM CaCl₂ and the

pH is adjusted 6.8-7.2. Finally, Tween-80 is added to 0.01% and the pH is adjusted to 5.5 for storage at -80°C .

Example 7

5 Experimental results

Subjecting the variants of the invention to the "Whole Blood Assay" revealed that the variants exhibit a significantly increased clotting activity (or reduced clotting time) as compared to rhFVIIa. The experimental results are compiled in Table 1 and in Fig. 1.

Table 1

Variant	Clotting time (Whole Blood Assay)	
	$t_{\text{variant}}/t_{\text{wt}}$	
rhFVIIa (reference)	1	
15 D196K	0.4	
D196N	0.4	
K341Q	0.4	
G237L	0.3	
20 G237GAA	0.3	

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. For example, all the techniques and apparatus described above may be used in various combinations. All publications, patents, patent applications, and/or other documents cited in this application are incorporated herein by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, and/or other document were individually indicated to be incorporated herein by reference in its entirety for all purposes.

CLAIMS

1. A variant of FVII or FVIIa, wherein said variant comprises at least one modification in a position selected from the group consisting of 196, 237 and 341 as compared to hFVII or hFVIIa (SEQ ID NO:1).
2. The variant according to claim 1, wherein said variant is a variant of hFVIIa.
3. The variant according to claim 1 or 2, wherein said variant comprises a modification in position 196 as compared to hFVII or hFVIIa (SEQ ID NO:1).
4. The variant according to claim 3, wherein said modification is a substitution.
5. The variant according to claim 4, wherein said substitution is D196K or D196N.
6. The variant according to claim 1 or 2, wherein said variant comprises a modification in position 237 as compared to hFVII or hFVIIa (SEQ ID NO:1).
7. The variant according to claim 6, wherein said modification is a substitution.
8. The variant according to claim 7, wherein said substitution is G237L.
9. The variant according to claim 6, wherein said modification is an insertion.
10. The variant according to claim 9, wherein said insertion is selected from the group consisting of G237GXX, G237GXXX and G237GXXXX, wherein X is any amino acid residue.
11. The variant according to claim 10, wherein X is selected from the group consisting Ala, Val, Leu, Ile, Gly, Ser and Thr.

12. The variant according to claim 11, wherein X is Ala.
13. The variant according to claim 12, wherein said insertions are G237GAA.
- 5 14. The variant according to claim 1 or 2, wherein said variant comprises a modification in position 341 as compared to hFVII or hFVIIa (SEQ ID NO:1).
15. The variant according to claim 14, wherein said modification is a substitution.
- 10 16. The variant according to claim 15, wherein said substitution is K341Q.
17. The variant according to any of claims 1-16, wherein said variant further comprises 1-15 amino acid modifications, such as 1-10 amino acid modifications; e.g. 1-5 amino acid modifications or 1-3 amino acid modifications.
- 15 18. The variant according to claim 17, wherein said further modifications are substitutions.
19. The variant according to claim 17 or 18, wherein at least one of said further amino acid substitutions is made in the Gla domain.
- 20 20. The variant according to claim 19, wherein said further substitution is made in a position selected from the group consisting of P10, K32, D33, A34 and combinations thereof as well as an insertion between A3 and F4.
- 25 21. The variant according to claim 20, wherein said further substitution is made in position K32.
22. The variant according to claim 21, wherein said further substitution is K32E.
23. The variant according to any of claims 19-22, wherein said further substitution is made in
30 position P10.

24. The variant according to claim 23, wherein said further substitution is P10Q.
25. The variant according to claims 19-24, wherein said further substitutions are made in
- 5 P10+K32+D33+A34 as well as insertion of an amino acid residue between A3 and F4.
26. The variant according to claim 25, wherein said further substitutions are
A3AY+P10Q+K32E+D33F+A34E.
- 10 27. The variant according to any of claims 19-26, wherein no modifications are made in residues
6, 7, 14, 16, 19, 20, 25, 26, 29 and 35.
28. The variant according to any of claims 1-27, wherein at least one amino acid residue
comprising an attachment group for a non-polypeptide moiety has been introduced or removed.
- 15 29. The variant according to claim 28, wherein at least one amino acid residue comprising an
attachment group for a non-polypeptide moiety has been introduced.
30. The variant according to claim 29, wherein at least one non-polypeptide moiety is covalently
- 20 attached to at least one of said attachment groups.
31. The variant according to claim 30, wherein said attachment group is a glycosylation site.
32. The variant according to claim 31, wherein said non-polypeptide moiety is a sugar moiety.
- 25 33. The variant according to claim 31 or 32, wherein the introduced glycosylation site is an *in vivo*
vivo glycosylation site.
34. The variant according to claim 33, wherein the *in vivo* glycosylation site is an *in vivo* O-
- 30 glycosylation site.

35. The variant according to claim 33, wherein the *in vivo* glycosylation site is an *in vivo* N-glycosylation site.

5 36. The variant according to claim 35, wherein said *in vivo* N-glycosylation site is introduced into a position comprising an amino acid residue having at least 25% of its side chain exposed to the surface (as defined in Example 1 herein).

10 37. The variant according to claim 36, wherein said *in vivo* N-glycosylation site is introduced into a position comprising an amino acid residue having at least 50% of its side chain exposed to the surface (as defined in Example 1 herein).

38. The variant according to any of claims 35-37, wherein said *in vivo* N-glycosylation site is introduced by a substitution selected from the group consisting of A51N, G58N, T106N, K109N,
15 G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N, D334N and combinations thereof.

39. The variant according to claim 38, wherein said *in vivo* N-glycosylation site is introduced by
20 a substitution selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N, D334N and combinations thereof.

40. The variant according to claim 39, wherein said *in vivo* N-glycosylation site is introduced by
25 a substitution selected from the group consisting of T106N, A175T, I205T, V253N, T267N+S269T and combinations thereof.

41. The variant according to any of claims 35-40, wherein one *in vivo* N-glycosylation site has been introduced by substitution.

42. The variant according to any of claims 35-40, wherein two or more *in vivo* N-glycosylation sites have been introduced by substitution.

43. The variant according to claim 42, wherein two *in vivo* N-glycosylation sites have been introduced by substitution.

44. The variant according to claim 42 or 43, wherein said *in vivo* N-glycosylation sites have been introduced by substitutions selected from the group consisting of A51N+G58N, A51N+T106N, A51N+K109N, A51N+G124N, A51N+K143N+N145T, A51N+A175T, A51N+I205T, A51N+V253N, A51N+T267N+S269T, A51N+S314N+K316T, A51N+R315N+V317T, A51N+K316N+G318T, A51N+G318N, A51N+D334N, G58N+T106N, G58N+K109N, G58N+G124N, G58N+K143N+N145T, G58N+A175T, G58N+I205T, G58N+V253N, G58N+T267N+S269T, G58N+S314N+K316T, G58N+R315N+V317T, G58N+K316N+G318T, G58N+G318N, G58N+D334N, T106N+K109N, T106N+G124N, T106N+K143N+N145T, T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, T106N+S314N+K316T, T106N+R315N+V317T, T106N+K316N+G318T, T106N+G318N, T106N+D334N, K109N+G124N, K109N+K143N+N145T, K109N+A175T, K109N+I205T, K109N+V253N, K109N+T267N+S269T, K109N+S314N+K316T, K109N+R315N+V317T, K109N+K316N+G318T, K109N+G318N, K109N+D334N, G124N+K143N+N145T, G124N+A175T, G124N+I205T, G124N+V253N, G124N+T267N+S269T, G124N+S314N+K316T, G124N+R315N+V317T, G124N+K316N+G318T, G124N+G318N, G124N+D334N, K143N+N145T+A175T, K143N+N145T+I205T, K143N+N145T+V253N, K143N+N145T+T267N+S269T, K143N+N145T+S314N+K316T, K143N+N145T+R315N+V317T, K143N+N145T+K316N+G318T, K143N+N145T+G318N, K143N+N145T+D334N, A175T+I205T, A175T+V253N, A175T+T267N+S269T, A175T+S314N+K316T, A175T+R315N+V317T, A175T+K316N+G318T, A175T+G318N, A175T+D334N, I205T+V253N, I205T+T267N+S269T, I205T+S314N+K316T, I205T+R315N+V317T, I205T+K316N+G318T, I205T+G318N, I205T+D334N, V253N+T267N+S269T, V253N+S314N+K316T, V253N+R315N+V317T, V253N+K316N+G318T, V253N+G318N, V253N+D334N, T267N+S269T+S314N+K316T,

T267N+S269T+R315N+V317T, T267N+S269T+K316N+G318T, T267N+S269T+G318N, T267N+S269T+D334N, S314N+K316T+R315N+V317T, S314N+K316T+G318N, S314N+K316T+D334N, R315N+V317T+K316N+G318T, R315N+V317T+G318N, R315N+V317T+D334N and G318N+D334N.

5

45. The variant according to claim 44, wherein said *in vivo* N-glycosylation sites have been introduced by substitutions selected from the group consisting of T106N+A175T, T106N+I205T, T106N+V253N, T106N+T267N+S269T, A175T+I205T, A175T+V253N, A175T+T267N+S269T, I205T+V253N, I205T+T267N+S269T and V253N+T267N+S269T.

10

46. The variant according to claim 45, wherein said *in vivo* N-glycosylation sites have been introduced by substitutions selected from the group consisting of T106N+I205T, T106N+V253N and I205T+T267N+S269T.

15 47. The variant according to any of claims 35-40, wherein three or more *in vivo* N-glycosylation sites have been introduced by substitution.

48. The variant according to claim 47, wherein three *in vivo* N-glycosylation sites have been introduced by substitution.

20

49. The variant according to claim 47 or 48, wherein said *in vivo* N-glycosylation sites have been introduced by substitutions selected from the group consisting of I205T+V253N+T267N+S269T and T106N+I205T+V253N.

25 50. The variant according to any of claims 30-49, wherein said variant has an increased functional *in vivo* half-life or an increased serum half-life as compared to rhFVIIa.

51. The variant according to any of claims 30-49, wherein said variant has an increased Area Under the Curve (AUC) when administered intravenous (AUC_{iv}), as compared to rhFVIIa.

30

52. The variant according to claim 51, wherein said variant is administered intravenous in rats.
53. The variant according to any of the preceding claims, wherein said variant is inhibited by the TFPI to a lesser extent than rhFVIIa.
54. The variant according to any of the preceding claims, wherein said variant further comprises at least one modification in a position selected from the group consisting 157, 158, 296, 298, 305, 334, 336, 337 and 374.
55. The variant according to claim 54, wherein said modification is a substitution selected from the group consisting of V158D, E296D, M298Q, L305V, K337A and combinations thereof.
56. The variant according to claim 55, wherein said substitutions are selected from the group consisting of V158D+E296D+M298Q+L305V+K337A,
V158D+E296D+M298Q+K337A, V158D+E296D+M298Q+L305V, V158D+E296D+M298Q,
M298Q, L305V+K337A, L305V and K337A.
57. The variant according to any of the preceding claims, wherein said variant further comprises at least one modification selected from the group consisting of L39E, L39Q, L39H, I42R, S43H, S43Q, K62E, K62R, L65Q, L65S, F71D, F71Y, F71E, F71Q, F71N, E82Q, E82N, E82K and F275H.
58. The variant according to any of the preceding claims, wherein said variant is in its activated form.
59. A nucleotide sequence encoding a variant as defined in any of claims 1-58.
60. An expression vector comprising a nucleotide sequence as defined in claim 59.

61. A host cell comprising a nucleotide sequence as defined in claim 59 or an expression vector as defined in claim 60.

62. The host cell according to claim 61, wherein said host cell is a gammacarboxylating cell
5 capable of *in vivo* glycosylation.

63. A pharmaceutical composition comprising a variant as defined in any of claims 1-58, and a pharmaceutically acceptable carrier or excipient.

10 64. The pharmaceutical composition according to claim 63, further comprising a vitamin K-dependent polypeptide different from any of the FVII or FVIIa variants as defined in any of claims 1-58.

65. A variant as defined in any of claims 1-58, or a pharmaceutical composition as defined in
15 claim 63 or 64, for use as a medicament.

66. Use of a variant as defined in any of claims 1-58 for the manufacture of a medicament for the treatment of a disease or a disorder wherein clot formation is desirable.

20 67. Use according to claim 66, wherein said disease or disorder is selected from the group consisting of hemorrhages, including brain hemorrhages, severe uncontrolled bleedings, such as trauma, bleedings in patients undergoing living transplantations, bleeding in patients undergoing resection and variceal bleedings.

25 68. Use according to claim 67, wherein said disease or disorder is trauma.

69. Use according to claim 68, wherein said disease or disorder is blunt trauma.

70. Use according to claim 68, wherein said disease or disorder is penetrative trauma.

71. A method for treating a mammal having a disease or a disorder wherein clot formation is desirable, comprising administering to a mammal in need thereof an effective amount of the variant as defined in any of claims 1-58 or the pharmaceutical composition as defined in claims 63 or 64.

5

72. The method according to claim 71, wherein said disease or disorder is selected from the group consisting of hemorrhages, including brain hemorrhages, severe uncontrolled bleedings, such as trauma, bleedings in patients undergoing living transplantations, bleeding in patients undergoing resection and variceal bleedings.

10

73. The method according to claim 72, wherein said disease or disorder is trauma.

74. The method according to claim 73, wherein said disease or disorder is blunt trauma.

15 75. The method according to claim 73, wherein said disease or disorder is penetrative trauma.

1/1

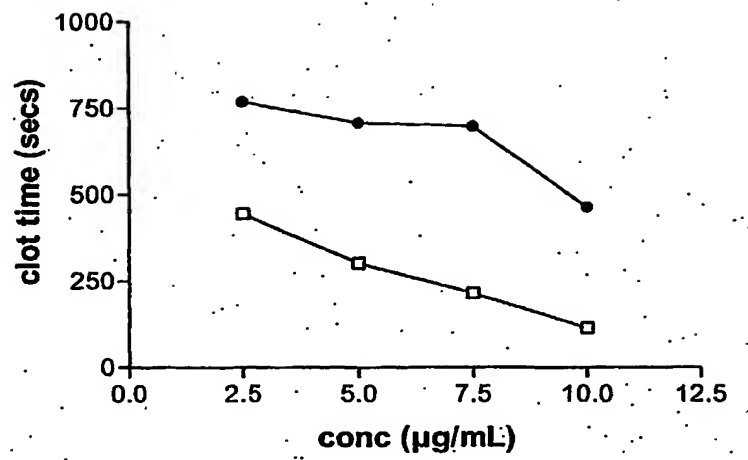


Fig. 1

SEQUENCE LISTING

SEQ ID NO:1

Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
 1 5 10 15
 Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
 20 25 30
 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
 35 40 45
 Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
 50 55 60
 Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
 65 70 75 80
 Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
 85 90 95
 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
 100 105 110
 Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
 115 120 125
 Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
 130 135 140
 Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
 145 150 155 160
 Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
 165 170 175
 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
 180 185 190
 His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
 195 200 205
 Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
 210 215 220
 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
 225 230 235 240
 His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
 245 250 255
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
 260 265 270
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu

275	280	285
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg		
290	295	300
Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser		
305	310	315 320
Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser		
	325	330 335
Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr		
	340	345 350
Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys		
	355	360 365
Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile		
	370	375 380
Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu		
	385	390 395 400
Leu Arg Ala Pro Phe Pro		
	405	

SEQ ID NO:2

atgggtcagcc aggcctctcgc cctcctgtgc ctgctcctgg ggctgcaggg ctgcctggct	60
gccgtcttcg tcaccagga ggaagccat ggcgtcctgc atcgccggcg ccgg gcc	117
Ala	
	1
aat gcc ttt ctg gaa gag ctc cgc cct ggc tcc ctg gaa cgc gaa tgc	165
Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu Cys	
5 10 15	
aaa gag gaa cag tgc agc ttt gag gaa gcc cgg gag att ttc aaa gac	213
Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys Asp	
20 25 30	
gct gag cgg acc aaa ctg ttt tgg att agc tat agc gat ggc gat cag	261
Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp Gln	
35 40 45	
tgc gcc tcc agc cct tgc cag aac ggg ggc tcc tgc aaa gac cag ctg	309
Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln Leu	
50 55 60 65	
cag agc tat atc tgc ttc tgc ctg cct gcc ttt gag ggg cgc aat tgc	357
Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn Cys	
70 75 80	

gaa acc cat aag gat gac cag ctg att tgc gtc aac gaa aac ggg ggc	405
Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly Gly	
85 90 95	
tgc gag cag tac tgc agc gat cac acg ggc acg aag cgg agc tgc cgc	453
Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys Arg	
100 105 110	
tgc cac gaa ggc tat agc ctc ctg gct gac ggg gtg tcc tgc acg ccc	501
Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr Pro	
115 120 125	
acg gtg gaa tac cct tgc ggg aag att ccc att cta gaa aag cgg aac	549
Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg Asn	
130 135 140 145	
gct agc aaa ccc cag ggc cgg atc gtc ggc ggg aag gtc tgc cct aag	597
Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro Lys	
150 155 160	
ggg gag tgc ccc tgg cag gtc ctg ctc ctg gtc aac ggg gcc cag ctg	645
Gly Glu Cys Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln Leu	
165 170 175	
tgc ggc ggg acc ctc atc aat acc att tgg gtc gtg tcc gcc gct cac	693
Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala His	
180 185 190	
tgc ttc gat aag att aag aat tgg cgg aac ctc atc gct gtg ctc ggc	741
Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu Gly	
195 200 205	
gaa cac gat ctg tcc gag cat gac ggg gac gaa cag tcc cgc cgg gtg	789
Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg Val	
210 215 220 225	
gct cag gtc atc att ccc tcc acc tat gtg cct ggc acg acc aat cac	837
Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn His	
230 235 240	
gat atc gct ctg ctc cgc ctc cac cag ccc gtc gtg ctc acc gat cac	885
Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp His	
245 250 255	
gtc gtg cct ctg tgc ctg cct gag cgg acc ttt agc gaa cgc acg ctg	933
Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr Leu	
260 265 270	
gct ttc gtc cgc ttt agc ctc gtg tcc ggc tgg ggc cag ctg ctc gac	981
Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu Asp	
275 280 285	
cgg ggc gct acc gct ctc gag ctg atg gtg ctc aac gtc ccc cgg ctg	1029
Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg Leu	
290 295 300 305	

atg acc cag gac tgc ctg cag cag tcc cgc aaa gtg ggg gac tcc ccc	1077
Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser Pro	
310 315 320	
aat atc acg gag tat atg ttt tgc gct ggc tat agc gat ggc tcc aag	1125
Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys	
325 330 335	
gat agc tgc aag ggg gac tcc ggc ggg ccc cat gcc acg cac tat cgc	1173
Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg	
340 345 350	
ggg acc tgg tac ctc acc ggg atc gtc agc tgg ggc cag ggc tgc gcc	1221
Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala	
355 360 365	
acg gtg ggg cac ttt ggc gtc tac acg cgc gtc agc cag tac att gag	1269
Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu	
370 375 380 385	
tgg ctg cag aag ctc atg cgg agc gaa ccc cgg ccc ggg gtg ctc ctg	1317
Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu	
390 395 400	
cgg gcc cct ttc cct tga taa	1338
Arg Ala Pro Phe Pro	
405	

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